REMARKS

On July 22, 2004, an RCE was filed with an amendment, since deemed non-compliant. SPE Andrew Wang has advised us that the non-compliant amendment was not entered, hence, the claim listing is relative to the claims as amended on August 5, 2003. Likewise, we have duplicated the July 22, 2004 remarks and attached fresh copies of the enclosures.

The additional claims fee was paid by credit card charge when we filed the RCE. We assume that the holding of non-compliance does not affect the financial processing, i.e., the July 22 payment was accepted, and will not be refunded. Hence, we don't need to make a replacement payment of the additional claims fee.

1. Claims

The present claims are directed to second and higher level glycopeptide libraries. Such libraries can be obtained by steps which comprise random glycosylation of existing carbohydrate structures of a library comprising glycopeptides.

The "level" of a glycopeptide library indicates the number of rounds of glycosylation employed in synthesizing the library.

P9, L2-9 states

> A first level library of a desired glycopeptide is created by primary glycosylation of the peptide with a single glycosyl donor or a mixture of donors. Reaction of a core peptide with a glycosyl donor, or mixture of donors, results in a library of randomly glycosylated glycopeptides. A first level library... can form the basis for generating higher level libraries.

Thus, a first level library is the result of glycosylating unglycosylated peptides. A glycosyl donor can

glycosylate such a peptide at any glycosylation site. For example, mono- and oligosaccharides can be N-linked to the peptide, by reaction with the NH_2 group on the side chain of Asn (or the NH_2-terminal of the peptide), or O-linked to the peptide, by reaction with the OH group on the side chain of Ser, Thr or hydroxylysine. P1, L21-28; P6, L14-20.

If the peptide were reacted with a single donor (i.e., random glycosylation were not employed), then the only way that a first level library could have the diversity implied by the term "library" would be if there were diversity in the amino acid sequence, e.g., by glycosylating a peptide library like that of P2, L9-30.

Random glycosylation is achieved by reacting the starting peptide(s) with a mixture of different glycosyl donors, such as galactosamine, N-acelylgalactosamine, and sialyl. P8, L9-21.

What, then, is a second or higher level library? According to P9, L10-25,

> A second level library is created by reacting one or more first level libraries with one or more further glycosyl donors. Prior to further reaction, unreacted glycosylation sites on the peptides may be blocked, e.g., by acetylation, in order to prevent these glycoforms from being eliminated from the library by being converted into different glycoforms. Following purification, the protecting groups of the carbohydrate structures on the glycoforms are selectively removed to create additional glycosylation sites on the existing carbohydrate structures. Random glycosylation with these additional donors further extends existing carbohydrate structures, thereby to create more complex glycopeptide structures. Higher level libraries are similarly created by reacting one or more second level or higher libraries with one or more further glycosyl donors.

When a glycosyl donor is reacted with a glycopeptide of a first level library, in theory it can react either (1) with a glycosylation site on the side chain of an unglycosylated amino acid, or (2) with a glycosylation site on a carbohydrate structure of the first level glycopeptide. If, prior to this second glycosylation reaction, the unreacted glycosylation sites on the amino acids are blocked, then possibility (1) is eliminated, and the second glycosylation can only extend the existing carbohydrate structure.

In reciting that peptides are "randomly glycosylated", applicants indicate that the starting peptide(s) are given the opportunity to react with the members of a mixture of glycosyl donors, and that some molecules are thus glycosylated. We do not mean to imply that every single peptide molecule must in fact be glycosylated; that would be contradictory to the teachings of P9, L12-13 to the effect that some glycosylation sites may remain unreacted.

Claim 32 has been rewritten to avoid product-by-process language, and to require O-linkage of the carbohydrate to the peptide scaffold.

New claim 47 recites that the peptide scaffolds are cyclic peptides.

New claim 48 recites that the peptide scaffold comprise at least one D-amino acid.

New claim 49 recites that the peptide scaffold comprises at least a four amino acid subsequence of the core protein of MUC1.

New claim 50 recites that one or more of the carbohydrate structures comprises sialic acid.

New claim 45 is a composite of the distinctive limitations of amended claim 32 and new claims 45-48, which are all dependent, through claim 46, on 45. However, if the

Examiner would prefer, we can cancel 45-46 and rewrite 32, 45, 46, 47 and 48 as independent claims. While claim 45 says that the library comprises glycopeptides with the same scaffold, claim 46 insists that all of the peptides, including glycopeptides, have the same scaffold. Please note that the libraries can include an unglycosylated peptide corresponding to the original peptide scaffold(s). Please also note that it is possible to mix together libraries, each made from a single scaffold, to obtain a new library featuring several different scaffolds, which is why claim 45 is not limited to a single scaffold.

Method-of-use claim 38, as amended, requires only screening for target-binding (i.e., "antibody-like") activity. Screening for biological activity has been moved to new claim 50, screening for immunostimulatory activity to new claim 51, and screening for competitive inhibition to new claims 52-53.

Table 1 (P. 10) sets forth the diversity of a first level library in which a single peptide scaffold, with 1-5 glycosylation sites, is randomly glycosylated with 1-5 different glycosylation donors, in such a manner that the degree of glycosylation of the peptides varies, from molecule to molecule, from 0 to 100%. Thus, with 5 glycosylation sites, as in SEQ ID NO:1, and a single glycosylation donor, the library has an overall diversity of 32, the library members consisting of one unglycosylated peptide, five singly glycosylated peptides, 10 doubly glycosylated peptides, 10 triply glycosylated peptides, five quadruply glycosylated peptides, and one quintuply glycosylated peptide. The library thereby consists of 31 glycopeptides and one unglycosylated peptide.

Claims 55-59 are based on Table 1, with 5 glycosylation sites and 1-5 carbohydrate structures. Note that peptides are considered "different" if they have different glycosylation

patterns, even if they have the same amino acid sequence. Also, "glycopeptides" are a subset of "peptides".

The diversity of a second level library will be equal to that of the first level library if the same sugar is added to all existing carbohydrate structures. More often, different sugars will be added, randomly, so its diversity will be greater than that of the starting first level library.

Claim 60 is based on table 1 with 2 sites and 2 structures. This corresponds to the first level library #3 of p. 25. Claim 61 is based on second level library #7 at P27, L35, which is derived from first level library #3.

Claim 62 is based on claim 1, as amended, of 09/842,873.

Claim 63's four amino acid limitation is based on the GSTA disclosure in Example 1.

Claims 64-65 refer to the peptide as being "derived" from a "cancer-associated mucin" (64) or from a MUC1 core protein (65). We interpret "derived" to allow the peptide to be a fragment of the core protein of the mucin (e.g., MUC1), since there is specific disclosure of two fragments of MUC1 (a 16 a.a. fragment at P11, L17-21, and a four amino acid fragment in Example 1 on p. 13). We also interpret "derived" as allowing replacement of L-amino acids with the corresponding D-amino acids, in accordance with the teachings of P6, L21-25, labeling of amino acids with UV-active or fluorescent labels per P6, L26-28, cyclization of the peptide per P6, L26, and lipidation per P6, L37-P7, L2. Naturally, it also allows for glycosylation.

Claims 66-69 and 72 are related to the above disclosures. Claims 70-71 are based on P5, L36-37.

Claims 73-74 refer to the component carbohydrate structure diversity, and are based on the number of different glycosyl donors in the reaction mixture, at least three (claim 73) or at least five (claim 74), per P8, L16, P9, L33. Claims

75-77 relate to the extent of the glycosylation. Claims 75 and 77 presuppose that all glycosylatable AAs are free, per P7, L4-5. Claim 76 formally describes the distribution of library members vis-a-vis degree of glycosylation which is inherent in the formula of P9, L29. See also P11, L21-34; P24, L2-12; P27, L17-P28, L9.

Claims 78-82 define, in various ways, the variation in glycosylation pattern among the glycopeptides of the library.

2. Prior Art Issues

Claims 32, 34-38 and 42-43 were rejected as anticipated by or obvious over Rao. Likewise claims 32 and 34-38 were rejected as anticipated by or obvious over (1) Vetter or (2) Schleyer. Finally, claims 32-37 were rejected as anticipated by or obvious over Frische et al. (J. Pept. Sci. abstract).

2.1. Rao creates a glycopeptide library using fucose-serine building blocks. No amino acid other than serine is glycosylated, and no sugar other than fucose is disclosed. Thus, there is no randomness in Rao's glycosylation step. Rather, the randomness is in the amino acids to which the Fuc-Ser is attached.

The Examiner, in support of the argument that Rao teaches random reaction, refers us to Rao col. 21, lines 44-49:

> In a similar manner as shown in Example VII, other peptide derivatives containing (multiple) carbohydrate residues are prepared to mimic the binding of SLex to selectin binding sites. Carbohydrate residues include mannose, glucose, N-acetylglucosamine, N-acetylgalactosamine, galactose and sialic acid.

However, this passage is fairly read as teaching replacement of fucose with one other carbohydrate selected from the recited group, not reaction of the peptide with a mixture of

all of the suggested carbohydrates. All discussions of randomization are in the context of the amino acid sequence, see, e.g., col. 8, lines 39-45.

The instant claims require a carbohydrate structure diversity which is manifestly absent from Rao. Rao does not obtain the "same library product", and does not suggest randomization of the carbohydrate content.

2.2. Vetter et al. (pp. 25-27) discloses the synthesis of glycoconjugate library of the form Ac-X-X-E(OAl)-X-P-resin, where Ac is acetyl, E is Glu, P is Pro, each X is randomly selected from a set of 18 side chain-protected AAs, and "OAl" is the allyl ester protecting group. First, a peptide library (diversity 18^4) was synthesized. Then it was converted into a glycopeptide library by removing the allyl ester and replacing it randomly with one of a set of 17 glycosylamines (P26, L29-31); these were mono- or disaccharides.

Thus, Vetter randomly glycosylated a glycosylation site on a "platform" (peptide) to create a first level library of glycosylated platforms, per step (a) of claim 1.

In Vetter's application, the disclosed method is to make N-linked glycopeptide (glycoconjugates) libraries. The glycosylation acceptor (preferably a peptide), has a free carboxylic acid (-COOH) as a side chain. In a naturally occurring peptide, the amino acids with free carboxylic acid side chain functionalities are Asp and Glu. This glycosylation acceptor is pre-activated, i.e., the free -COOH side chain is converted into a reactive pentafluorophenyl (Pfp) ester. Then the glycosyl donor, a carbohydrate with an amine (-NH2) attached to the reducing carbon, i.e., a glycosylamine, is reacted with the activated ester to form an amide linkage between the carbohydrate and the carboxyl side chain of the peptide (i.e., carbohydrate-**N**H-CO-peptide).

The effect of the last step is to convert Asp (side chain

-CH_2COOH) or Glu (side chain -CH_2CH_2COOH) into Asn (side chain -CH_2CONH_2) or Gln (side chain -CH2Ch2CONH2), respectively. Since the carbohydrate is then attached to the N of the resulting Asn or Gln, it is considered to be N-linked.

Vetter's glycosylation donors will not react with the hydroxy functions of Ser or Thr to form O-linked moieties; Vetter only generates N-linkages.

Claim 32, as amended, contemplates reaction of a peptide (the glycosylation acceptor) having at least one free hydroxyl group (i.e., an O-linkable glycosylation site) with a mixture of glycosylation donors, so that at least some of the peptides are O-glycosylated. That is, the result of the reaction is to form some glycopeptides in which the sugars are O-linked to the core peptide. (If the peptide comprises amino acids with free amino groups, the donors can also react at these sites to form N-linked glycopeptides.) In either case, the amino acid sequence remains unaffected.

Our method and Vetter's produce different libraries. Thus, if the initial core peptide is VDTA, Vetter will obtain just VDTA or VN*TA (where * denotes glycosylation), while we obtain VNTA, VNT*A, VN*TA and VN*T*A.

If the core peptide were PDTRP (a Muc1 epitope), we would obtain PDTRP or PDT*RP, while Vetter would obtain PDTRP or PN*TRP.

If the core peptide were NQN[1], we obtain 8 different species (NQN, N*QN, NQ*N, NQN*, N*W*N, N*QN*, NQ*N*, and N*Q*N*). Vetter could make a library which contained N*Q*N*, but he would have to start with the core peptide DED, and he would then also obtain N*ED, DQ*D, DEN*, N*Q*D, N*EN*, and

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[1] This core peptide is outside claim 32, but is cited to show how Vetter's chemistry results in N-linked glycopeptides with altered sequences. A core peptide like NQNT could generate a library within claim 32.

DQ*N*.

2.3. Claims 32-37 stand rejected as anticipated by or obvious over Frische et al. abstract (J. Peptide Sci.). In general, we think it inadvisable for the Office to cite abstracts; the entire article should be made of record. A copy of the article is enclosed.

Frische et al. studied the fragment 67-76 (VITAFNEGLK) of CBA/J mouse hemoglobin, which binds to CBA/J mouse MHC class II molecule E^k.

Frische et al. first synthesized the series of peptides #s28-67 set forth in Table 5. These peptides are single substitution mutants[2] which differ from the native fragment in that a single amino acid is replaced by Ser, Thr, glycosylated Ser or glycosylated Thr. The glycosylated AAs of Table 5 are all glycosylated with D-GalNAc. Frische systematically replaced each amino acid of the 10 a.a. fragment in this manner ("scanning mutagenesis"), thereby obtaining 40 different peptides, 20 of which were glycosylated.

Frische et al. discovered that the Hb (67-76) peptide became immunogenic when position 72 was Tn (i.e., D-GalNAc)-glycosylated.

Hence, Frische prepared a second series of 12 peptides, set forth in Table 6. The position 72 amino acid was Ser, Thr or Gln, and it was glycosylated with β-D-Gal, β-D-GalNAc, α-L-FUc, β-L-Fuc, β-D-GlcNAc, α-D-Man, α-D-Glc(1-4) β-D-Glc, α-D-Glc (1-4)α-D-Glc(1-4)-β-D-Glc, or α-D-GlcNAc. Only 12 of the possible combinations were explored. When position 72 was Ser or Thr, the carbohydrate was O-linked, and when position 72 was Asn, the carbohydrate was N-linked.

The Table 6 peptide series is not a library as each glycopeptide was individually synthesized on its own column of

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[2] Peptide #40 is actually the natural fragment.

a 20 column manual synthesizer. There was no randomization because at no time was a mixture of glycosyl donors used as a reactant. Hence, there is no anticipation of the instant claims.

If Frische et al.'s peptides 68-79 were mixed togther, this could be considered a library, but it still would not anticipate because the library members would not have the same peptide scaffold. There would instead be three different scaffolds.

Frische et al. does not provide motivation to mix together only the glycopeptides which have the same core peptide sequence, such as his peptides #'s 68-71.

Frische is further distinguished by several dependent claims, e.g., claim 75, which requires that the peptide scaffold comprise a plurality of glycosylation sites and that at least one of the molecules in the library be glycosylated at all of those sites.

3. Utility/Enablement (OA pp. 2-5)

The Examiner asserts that the library does not have a specific and well established utility, even as a "research tool", because

(1) libraries are not one of the "research tools" enumerated in MPEP 2107;

(2) since there is no claimed structure for the library, the library cannot be used for screening purposes.

(3) the issuance of patents on third party combinatorial libraries is irrelevant as each case must be judged omits own merits.

(4) the library is useful only to the extent that an individual compound in the library has the sought for binding activity.

We answer these points as follows:

(1) MPEP 2107 does not purport to provide an exhaustive list of "research tools". Moreover, it does list "screening assay". An assay is a method, not a "tool". So by reference to "screening assays", it really means assay kit components. A library could readily be a component of a screening assay kit, and indeed combinatorial libraries are sold as such.

(2) The synthesizer of a given library would know which peptide scaffold, was used, and which glycosylation sites were exposed to which glycosylation reagents. Thus, such user would know the "structure space" explored by the library.

Even a user who did not synthesize the library could analyze several members of the library and make deductions as to the nature of the peptide scaffolding and glycosyl donors used.

If a library glycopeptide bound to a target, its structure could be determined without undue experimentation by mass spectrometry, as disclosed at P12, L17-19.

(3) The courts have given weight to third party patents when they occur in sufficient numbers to imply existence of a consensus in the art. The following cases illustrate the relevance of prior patents:

> Ex parte Brian, 118 USPQ 242, 245, (POBA 1958) (past practice of office in accepting definiteness of "fingerprint" claims);
>
> In re Chakrabary, 596 F.2d 952, 985-86 (CCPA 1979) (product claims reciting microorganisms previously treated as directed to statutory subject matter);
>
> Andrew Corp. v. Gabriel Electronics, Inc., 6 USPQ 2010, 2012 (Fed. Cir. 1988) (term "substantially" is "ubiquitous" in patent claims and therefore considered definite);
>
> In re Cortright, 49 USPQ2d 1464 (Fed. Cir. 1999) (Construction of "restore hair growth" for purpose of determining both §112 enablement and §101

utility; prior art references may be indicative of how a claim term will be interpreted by those of ordinary skill in the art);

Vitronics Corp. v. Conceptronic Inc., 39 USPQ2d 1573, 1578-9 (Fed. Cir. 1996) (prior art used to demonstrate how a disputed term is used by those skilled in the art, and indeed is more objective and reliable than post-litigation expert opinion testimony);

Pioneer Hi-Bred International v. J.E.M. Ag Supply Inc., 49 USPQ2d 1813, 1819 (N.D. Iowa 1998) (issuance of Boehm USP 2,048,056 in 1936 is evidence that "in those instances where inventors showed they could define a reproducible plant meeting the limits of §112, plant patents were issued under §101".)

A large number of patents have issued with broad combinatorial compound library claims of some kind. In Appendix 1, we provide a sampling of these patents. This non-exhaustive listing is mainly of claims to combinatorial libraries of peptides, proteins, peptoids and polynucleotides, as the diversity of the library is more immediately apparent than is the case for a claim to, e.g., a benzodiazepine library. Clearly, the examiners of these patents thought that the claimed libraries had utility and were enabled for their full scope, even though they were not limited (as the present examiner proposes) to close analogues of a specific molecule already known to have a particular activity.

There are a far larger number of patents with broad claims to methods of making or using combinatorial libraries. (A few examples are given in Appendix 2.) The standard of enablement utility for product claims is no more stringent than that for method claims. The issuance of these patents implies that the libraries have utility, because a method of making or using a useless product would itself lack utility. They, too, must be deemed fully enabled, even though they are

not limited to specific scaffolds.

(4) Actually, while it is certainly desirable that a library have a "hit", useful information is extracted even if there are no "hits". If, for example, MUC1 glycosylated with Tn and TF had no "hits", then the skilled worker would be discouraged from synthesizing and testing (1) the individual glycopeptides expected to be in the library, and (2) individual glycopeptides similar to those of (1) above. Thus, future testing could be rechanneled into more fruitful directions. And it is much less trouble to combinatorially synthesize and screen a library of 10^6 glycopeptides than to process them individually.

3.1. The first enablement rejection (bottom of OA p. 5) is predicated on the alleged lack of 35 USC 101 utility and hence is overcome by the argument in the last section.

4. Enablement Issue

Another enablement rejection is stated at OA pp. 7-8.

The Examiner concedes that the specification is enabling for (1) generating a glycopeptide library using "mucin 1 (MUC1) as the core protein", and (2) screening that library for compounds with "inhibitory activity", but questions enablement for more broady claimed libraries.

The specification clearly contemplates use of platforms other than MUC1. Peptides, and in particular the core proteins of cancer associated mucins, are of particular interest (P1, L9-13). There is no reason to believe that other peptides would be more difficult to randomly glycosylate then would MUC1. Many different natural glycopeptides are known in the art. Of course, the starting peptide must feature at least one glycosylation site.

Platforms are discussed further at P5, L30-P7, L11. The specification says that the "platform" can be a peptide and,

if so, that it may be linear or cyclic. It is also clear that the peptide may be composed of l- or d-amino acids. Reference is made to hydrophobic amino acids at P6, L35-37, and to glycosylatable amino acids at P1, L21-28.

Several specific platforms other than MUC1 are disclosed. The first is Tn antigen. Since Tn antigen is GalNAc-O-serine, the platform is just Ser per se. TF antigen has the same platform (P7, L30-34).

Another platform of interest is the one shown in Fig. 4. This is a peptide with an unusual bridged structure. See also the peptoid (-CH_2CH_2- linkage) of Fig. 5. We also mention OSM and CA27.29 at P11, L11-14.

Please also see our discussion of Appendices 1 and 2 in the prior section. Claims were issued, hence deemed fully enabled, even though they were not limited to specific scaffolds.

5. Description Issues

5.1. The Examiner maintains description rejection "B" from pp. 5-6 of the office action of May 21, 2001:

> The specification fails to provide an adequate description of the components of a library that has competitive inhibitory, immunostimulatory or antibody activity. The specification provides a generalized statement as broad as the claimed invention. The components of the library are merely recited to be a glycopeptide without defining the structure. Without defining the structure, the glycopeptide comprises ever conceivable possible combinations of diverse structures defined only by the different opposite functions such as inhibition, immune stimulation or antibody activity.

The immediate office action (pp. 6-7) adds:

> Applicants argue that armed with

> applicants' disclosure of how to produce a combinatorial glycopeptide library, a skilled artisan readily could select platforms and carbohydrates to produce a library of compounds likely to contain some with of the recited activities.
>
> In response, applicants fail to specifically point out the relevant section applicants are relying on. Thus, it is not clear as to the method that a skilled artisan needs to employ to accomplish which of the different opposing activities the library possesses.

If we understand this rejection correctly, the underlying reasoning is this:

(1) the disclosed activities ("competitive inhibitory, immunostimulatory or antibody activity" or "inhibition, immunostimulation or antibody activity") are "opposite", i.e., unlikely to be possessed by the same compound.

(2) the structures of the library members are defined only by a general compound class (glycopeptide) and a function, and, since the disclosed functions are "opposite", the encompassed structures are diverse.

When a claim is to an individual peptide, and the utility is dependent on the sequence, there is reason to say that "description" requires recitation of the sequence. However, the utility of a library is dependent on its diversity.

Claim 32 requires that for a given library, all of the (glyco)peptides have the same peptide scaffold. The only variation, consequently, is with regard to which carbohydrate structures are attached to that scaffold and where. Moreover, the choice of scaffold (core peptide) limits the possible location of the carbohydrate structures, as they must have been attached as a result of glycosylation of a glycosylation site (i.e., Ser, Thr, Asn, Gln in the case of a genetically

encoded peptide) of the starting peptide.

With regard to the issue of "functions", we respectfully point out that many the instant claims, including 32, are to libraries per se. Hence, it is not necessary that the claimed libraries possess all of the disclosed functions. The only examined method of use claim was 38.

With regard to an antibody like function, we interpret that as meaning that the peptide specifically binds a target molecule. The experience with peptide libraries is that with a sufficiently diverse libraries, one or more binding peptides can be found for any target of interest. Certainly, if the core peptide of our glycopeptide library binds a target of interest, there is a reasonable expectation that one or more of our glycopeptides will also bind that target.

That said, in our opinion the library members do not have to actually offer the desired function, merely be screenable for it. If the library does not contain any members with a desired function, then that tells the skilled worker to avoid those members, and perhaps also closely related molecules. Screening a combinatorial library allows one to quickly eliminate potential binding molecules which don't work.

Competitive inhibitory activity is also commonly encountered in peptide libraries. That is, there is a known target-binding ligand, one or more peptides will competitively inhibit the binding to the target.

It is reasonable to expect that if the core peptide has immunostimulatory activity, and the library is sufficiently diverse, one or more of the library members will possess at least some of that activity. If they don't, then if some of the members bind the active site, then by occluding the site so the core peptide cannot act, then they demonstrate a competitive inhibitory function.

We have amended method claim 38 to require screening

merely for target binding (i.e., antibody-like) activity. When such binding activity, in vivo, has a biological effect, then one is in effect screening for a biological activity (see new claim 51). Immunostimulatory activity (new claim 52) is simply one form of biological activity. Binding, whether in vivo or in vitro, can competively inhibit a known ligand (new claims 53-54).

We again remind the Examiner that Appendices 1 and 2 demonstrate the general acceptance of combinatorial library claims as satisfying description even though there is a random component.

5.2. We do appreciate the examiner's concession that there is description for a glycopeptide library derived from the MUC1 protein.

The Examiner's attention is respectfully directed to claims 49, 45 (III), 65, 67, 68, 69 and 72.

5.3. The Examiner also asserts that applicants have acquiesced in the description rejection relating to "adhesion ligands for bacterial receptors expressed on human cell surface antigens".

The claim erroneously referred to "adhesion ligands for bacterial receptors expressed on human cell surface antigens. The adhesins are bacterial proteins, and they bind to carbohydrate receptors expressed on human cell surface antigens (which may be glycolipids or glycoproteins). Claims 30 and 32 have been amended accordingly.

It is well known in the art that bacteria bind to mammalian cells, and that such binding is the result of the binding of bacterial proteins (adhesins) to cell surface receptors. Some of these receptors are carbohydrate in character.

A good review of adhesin-carbohydrate receptor interactions appears in Karlsson, et al., "Microbial

Interaction with Animal Cell Surface Carbohydrates", APMIS Suppl. 27:71-83 (1992) (copy enclosed). According to Karlsson, the principal bacterial adhesin targets are

lactosylceramide (R1-Galβ4Glc-R2)
galabiose (R1-Galα4Gal-R2)
sialic acid (R1-NeuAC-R2).

Karlsson notes that E. coli class II adhesin binds to globoside (GalNAcβ3 Galα4Gal β4 Glc β Cer), and E. coli Class III adhesin to Forssman glycolipid (GalNAc α3 GalNAc β3 Gal α4 Gal β4 Glc β cer) and to blood group A globoside (GalNAc α3 (Fucα2) Gal β3 Gal NAc β3 Gal α 4 Gal β4 Glc β Cer).

See also Beachey, "Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface", J. Infect. Dis. 143(3):325-45 (1981) and Feizi, "Glycoprotein oligosaccharides as recognition structures", Ciba Found. Symp. 145:62-79 (1989) (copies enclosed).

The component carbohydrate structures (i.e., the individual sugars) forming the carbohydrates receptors for bacterial adhesins are, not surprisingly, the same as those which are incorporated into cancer-associated mucins. Obviously, they are the typical sugars of mammalian glycoproteins: Gal, GalNAc, Glc, GlcNAc, Fuc, Sialic acid (NeuAc) and Man.

At the time of filing of provisional application 60/056,240 (1997), the art was well aware of the structural character of the adhesin receptors contemplated by claim 30, and patent law does not require specific identification of those receptors unless the receptors are expressly claimed.

6. Definiteness Issues

The Examiner asserts that the claims are indefinite because the identities of the compounds comprising the library

are unknown, and that the identification is feasible by reference to a specific parent compound.

However, applicants plainly disclose that a variety of different parent compounds can be used — although, for any given library, there is just one parent compound.

Claim 32, as amended, recites that (1) there is only one peptide scaffold, (2) the scaffold comprises an O-linkable glycosylation site, and (3) the carbohydrate structure components are associated with human cancer mucins or with the human cell surface carbohydrate receptors recognized by bacterial adhesins.

We again remind the Examiner that Appendices 1 and 2 demonstrate a consensus that claims to libraries are definite even though the recited molecules have a random element.

7. Objection

The examiner objects to the phrase "combinatorially-generated glycopeptide library". Combinatorial generation of a glycopeptide library is clearly disclosed at P5, L16-23.

8. Finality

On May 28, the Examiner advised Counsel that presentation of any claim not identical in scope to one already pending would be deemed to raise a new issue requiring further consideration and search. Since this amendment does present such claims, the next action cannot be made final.

Respectfully submitted,

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Enclosures
-Appendix 1
-Appendix 2
-Karlsson (1992)
-Beachey (1981)
-Feizi (1989)

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APPENDIX 1: COMBINATORIAL LIBRARY PRODUCT CLAIMS

Schatz 5,270,170

26. A random peptide library composed of at least 10.sup.6 different members, wherein each member is a plasmid that encodes a lac repressor DNA binding protein and contains a lacO binding site for the DNA binding protein and a coding sequence for a random peptide inserted into the coding sequence of the DNA binding protein such that the resulting vector encodes a fusion protein that is composed of the DNA binding protein and the random peptide; and wherein each different member differs from other members with respect to the sequence of the random peptide.

Rutter 5,266,684

1. A predetermined mixture of peptides containing 8,000 or more different peptides of distinct, unique and different amino acid sequences, wherein the presence of each peptide in the mixture is predetermined, each peptide is present in the mixture in retrievable and analyzable amounts and the mixture includes at least one biologically active peptide in a retrievable and analyzable amount.

Kauvar 5,340,474

1. A panel for determining binding ability of a paralog to a target substance consisting essentially of individual candidate paralogs wherein said individual candidate paralogs have systematically varied values of at least two parameters over a maximal range to obtain maximal diversity in binding ability over the panel, each of which parameters determines the ability of the paralog to bind to other substances and/or wherein the combination of said parameters determines the ability of the paralog to bind to other substances.

Ladner 5,403,484

1. A virus bearing on its outer surface a chimeric binding protein, said protein comprising (i) a proteinaceous binding domain, other than a single chain antibody, which is sufficiently stable in structure to have a melting point of at least 40.degree. C., and which binds to a target:, other than the variable domain of an antibody, sufficiently strongly so that the dissociation constant of the binding domain: target complex is less than 10.sup.-6 moles/liter, and (ii) at least a functional portion of a coat protein of said virus, said portion acting, when the chimeric protein is produced in a suitable host cell, to

cause the display of the chimeric binding protein or a processed form thereof on the outer surface of the virus, said binding domain being capable of binding to a target material which said coat protein does not preferentially bind, said binding domain being foreign to the native coat proteins of said virus.

17. A library of virus according to claim 1, said library collectively displaying a plurality of different binding domains.

26. A chimeric binding protein comprising (i) a proteinaceous binding domain, other than a single chain antibody, which is sufficiently stable in structure to have a melting point of at least 40.degree. C., and which binds to a target, other than the variable domain of an antibody, sufficiently strongly so that the disassociation constant of the binding domain: target complex is less that 10.sup.-6 moles/liter, and (ii) at least a functional portion of a coat protein of a virus, said portion acting, when the chimeric protein is produced in a suitable host cell, to cause the display of the chimeric binding protein or a processed form thereof on the outer surface of the virus, said binding domain being capable of binding to a target material which said coat protein does not preferentially bind, said binding domain being foreign to the native coat proteins of said virus.

44. A fusion protein comprising (a) a carrier protein moiety essentially corresponding to a mature gene III protein of a filamentous phage, said carrier protein moiety acting, when the fusion protein is produced in a suitable host cell infected by the phage, to cause the display of the fusion protein or a processed form thereof on the surface of the phage, and (b) a foreign peptide or protein coupled to the amino terminal of said carrier protein moiety.

45. A recombinant filamentous phage bearing a fusion protein according to claim 44, upon its outer surface, said carrier protein moiety being integrated into the coat of the phage, said foreign peptide or protein being capable of binding specifically to a target which said phage does not specifically bind and being of an amino acid sequence foreign to the coat proteins native to said phage.

46. A library of recombinant phage according to claim 45, said library displaying a plurality of different foreign peptides or proteins.

47. A fusion protein comprising (a) at least a functional portion of a mature gene VIII protein of a filamentous phage, said portion acting, when the fusion protein is produced in a suitable

host cell infected by the phage to cause the display of the fusion protein or a processed form thereof on the surface of the phage, and (b) a foreign peptide or protein coupled to said functional portion of said mature gene VIII protein.

48. A recombinant filamentous phage bearing a fusion protein according to claim 47, upon its outer surface, said functional portion of the gene VIII protein being integrated into the coat of the phage, said foreign peptide or protein being capable of binding specifically to a target which said phage does not specifically bind and being of an amino acid sequence foreign to the coat proteins native to said phage.

49. A library of recombinant phage according to claim 48, said library displaying a plurality of different foreign peptides or proteins.

Ladner 5,571,698

67. A variegated population of replicable genetic packages, each package including a nucleic acid construct coding for a chimeric potential binding protein, each said construct comprising DNA encoding (i) a potential binding domain which is a mutant of a predetermined parental binding domain, and (ii) an outer surface transport signal for obtaining the display of the potential binding domain on the outer surface of the genetic package, wherein said initial binding domain is not a single chain antibody and is not identical to or substantially homologous with a binding domain natively associated with said transport signal, and wherein said variegated population of genetic packages collectively display a plurality of different potential binding domains, the differentiation among said plurality of different potential binding domains occurring through the at least partially random variation of one or more predetermined amino acid positions of said parental binding domain to randomly obtain at each said position an amino acid belonging to a predetermined set of two or more amino acids, the amino acids of said set occurring at said position in predetermined expected proportions.

68. A variegated population of DNA molecules encoding chimeric binding proteins, each said chimeric binding protein comprising (i) a binding domain, and (ii) at least a segment of an outer surface protein of a cell or virus, said segment acting to cause the display of the chimeric binding protein or a processed form thereof on the outer surface of the cell or virus, said binding domain being capable of binding to a target material to which said outer surface protein is not capable of preferentially binding, wherein said variegated population of DNA molecules

encode chimeric binding proteins which collectively include a plurality of different binding domains, the differentiation among said plurality of different potential binding domains occurring through the at least partially random variation of one or more predetermined amino acid positions thereof to randomly obtain at each said position an amino acid belonging to a predetermined set of two or more amino acids, the amino acids of said set occurring at said position in predetermined expected proportions.

81. A library of display phage which each displays on its surface, as a result of expression of a first phage gene, one or more copies of a particular chimeric coat protein, each chimeric coat protein comprising a potential binding domain which is a mutant of a known protein domain foreign to said phage, said library collectively displaying a plurality of potential binding domains, wherein the differentiation among said plurality of different potential binding domains occurs through the controlled random variation of one or more predetermined amino acid positions of said known domain to randomly obtain at each said position an amino acid belonging to a predetermined set of two or more amino acids, the amino acids of said set occurring at said position in predetermined expected proportions, contacting said library of phage with the target material, and separating the phage on the basis of their affinity for the binding protein target material, wherein the differentiation among said plurality of different potential binding domains occurs through the at least partially random variation of one or more predetermined amino acid positions of said known domain to randomly obtain at each said position an amino acid belonging to a predetermined set of two or more amino acids, the amino acids of said set occurring at said position in predetermined expected proportions, and in substantially all sets the ratio of the frequency of occurrence of the most favored amino acid to that for the least favored amino acid is less than 2.6, characterized in that, at at least one such position, the predetermined set consists of less than all twenty different genetically encodable amino acids, but includes three or more of the classes of genetically encodable amino acids.

82. A library of display phage which each displays on its surface, as a result of expression of a first phage gene, one or more copies of a particular chimeric coat protein, each chimeric coat protein comprising (a) a potential binding domain which is a mutant of a known protein domain foreign to said phage, as well as (b) at least a functional portion of a coat protein native to said phage, said library collectively displaying a plurality of potential binding domains, wherein said chimeric coat protein further comprises a linker peptide which is specifically

cleavable by said site-specific protease, said linker peptide being positioned inbetween (a) said potential binding domain, and (b) said native coat protein sequence, whereby (a) may be freed from (b).

Stuart 5,683,899

1. A panel comprising two or more heterokaryons wherein each of said heterokaryons produces a different variant of a multimeric protein and each heterokaryon is formed by fusing a first and a second fungal parent strain, wherein said heterokaryon requires the presence of both fungal parent nuclei for survival, said first and said second parent fungal strain each contain an exogenously supplied nucleic acid molecule that encodes a variant of a subunit of a multimeric protein and where said first and said second parent strains are homozygous for all heterokaryon compatibility alleles.

Still, 6,503,759

12. A library of compounds, each compound in the library being produced by a single reaction series and being bound to an individual solid support, each solid support having bound to it a combination of at least four distinguishable identifiers which differ one from another, at least one of which prior to binding to the solid support is encompassed by the formula:

F.sup.1 --F.sup.2 --(C--W)

wherein

F.sup.1 is CO.sub.2 H;

F.sup.2 is ##STR76##

A is --O--, --OC(O)O--, --OC(O)--; or --NHC(O)--;

C is C.sub.1 -C.sub.20 alkylene optionally substituted by 1-40 F, Cl, or Br,

W is --H, --OH, or amino;

R.sup.1 is H or C.sub.2 -C.sub.6 alkyl;

each of said identifiers being bound to said solid support either directly or indirectly or through other than a tag component of another identifier, wherein each identifier or combination

thereof encodes information as to a particular choice as a particular stage in the reaction series.

Lam 5,650,489

1. A library comprising a multiplicity of solid phase supports, wherein a single peptide species is attached to each solid phase support, and in which the library is prepared by a method comprising repeating k times, wherein k is at least three, the steps of:

(i) providing at least two aliquots of a solid phase support said supports having an N reactive site;

(ii) separately introducing a species of subunit of the peptides to each of the aliquots of solid phase supports such that a different subunit is introduced into each aliquot, said subunit having an N-blocking group and at least one of said subunits having a protecting group or a plurality of protecting groups;

(iii) completely covalently coupling the subunit to substantially all the reactive sites of the solid phase support;

(iv) thoroughly mixing and removing the N-blocking groups of the aliquots of the solid phase supports whereby an N reactive site is provided; and after repeating steps ii through (iv) k times, a final step of removing any protecting groups such that the deprotected peptides remain covalently attached to the solid phase supports.

12. The library of claim 11 comprising at least about 2,500,000 species.

Valerio 5,627,210

1. A compound of the formula: ##STR6## where R.sub.1, R.sub.2, and R.sub.3 are each independently groups of the formula --C(O)R, where

R is an **organic radical**;

x, y, and z are each independently 1, 2, 3, or 4;

R.sub.4 and R.sub.5 are each independently alkyl, alkenyl, aryl, aralkyl, acyl, amino, hydroxy, alkoxy, aryloxy, aryl-alkoxy, heterocyclyl, or H.

Thompson, 5,824,485

1. A combinatorial gene expression library, comprising a pool of expression constructs, each expression construct containing one or more cDNA or genomic DNA fragments, wherein the cDNA or genomic DNA fragments in the pool of expression constructs are derived from a plurality of species of donor organisms, and wherein the cDNA or genomic DNA fragments in each expression construct are operably-associated each with one or more regulatory regions that drives expression of genes encoded by the cDNA or genomic DNA fragments in an appropriate host organism.

Simon 5,811,387

1. A mixture of at least five non-homopolymeric polymers of differing sequences having a selected number of monomer units, said polymers selected from the group consisting of compounds of the general formula: X.sub.a --(NR--CH.sub.2 --CO).sub.n --X.sub.b, X.sub.a --(O--CHR--CO).sub.n --X.sub.b, X.sub.a --(NH--CHR--CS).sub.n --X.sub.b, X.sub.a --(NOH--CHR--CO).sub.n --X.sub.b, X.sub.a --(O--CHR--CH.sub.2 --CO).sub.n --X.sub.b, X.sub.a --(NH--CHR--CH.sub.2 --SO.sub.2)n--Xb, Xa--(NR--CH.sub.2 CH.sub.2 --SO.sub.2).sub.n --X.sub.b, X.sub.a --(NR--CH.sub.2 CH.sub.2 --NHCO).sub.n --X.sub.b and X.sub.a --(NR--CH.sub.2 CH.sub.2 --OCO).sub.n X.sub.b, wherein,

each R is a side chain which is independently alkyl of 2-6 carbon atoms, haloalkyl of 1-6 carbon atoms, alkynyl of 2-6 carbon atoms, cycloalkyl aryl of 6-10 carbon atoms, aryl-alkyl of 7-12 carbon atoms, substituted with 1-3 radicals independently selected from halo and nitro and hydroxy, aminoalkyl of 1-6 carbon atoms, hydroxyalkyl of 1-6 carbon atoms, carboxy, carboxyalkyl of 2-6 carbon atoms, carboalkoxy-alkyl of 3-10 carbon atoms, carbamyl, carbamylalkyl of 2-6 carbon atoms, guanidino, guanidinoalkyl of 1-6 carbon atoms, mercapto, mercaptoalkyl of 1-6 carbon atoms, alkylthioalkyl of 2-10 carbon atoms, imidazolyl, imidazolylalkyl of 4-10 carbon atoms, piperidyl, piperidylalkyl of 5-10 carbon atoms, indolyl, or indolylalkyl of 9-15 carbon atoms:

n is an integer from 2 to 50, inclusive; and

X.sub.a and X.sub.b are each independently H, lower alkyl, lower aryl, aralkyl, lower acyl, a polypeptide of 1-100 amino acids, or an effector molecule capable of exhibiting biological activity.

Sepetov 5,846,841

1. A library consisting of a multiplicity of sets, each said set comprising a multiplicity of species of test ligands, in which:

a) each species of test ligand of the library comprises a linker and a multiplicity of monomers, wherein:

i) said monomers are selected from a multiplicity of species of monomers; and

ii) said linker connects the monomers of a species of test ligand to a solid phase support particle or to an identifiable location of a solid support;

b) each monomer is disposed in one of a predetermined number of motif positions or in one of a predetermined or variable number of nonmotif positions, wherein:

i) the motif and non-motif positions have an ordered sequence; and

ii) there are a plurality of motif positions in each set of the library;

c) all species of test ligands of a particular set have:

i) a constant number of nonmotif positions and an identical, single ordered sequence of motif and nonmotif positions;

ii) an identical, single species of monomer, selected from a plurality of species of monomer, at each of the predetermined number of motif positions; and

iii) one of a plurality of species of monomers at each of the constant number of nonmotif positions;

d) all species attached to a particular solid phase particle or to a particular identifiable location of a solid support are encompassed by a single set; and

e) the library is a complete collection of sets in which all possible particular ordered

(i) sequences of the motif positions and nonmotif positions or

(ii) sequences of motif positions and intervals of nonmotif positions are represented.

Ruth, 5,817,786

1. A composition of single-stranded oligonucleotides, wherein the sequence of each of said oligonucleotides is substantially identical to the sequence of each of the other oligonucleotides in said composition,

wherein each of said oligonucleotides has a sequence length of not more than approximately 200 nucleotides,

wherein said oligonucleotides are prepared from nucleotide monomers, at least one of which has a linker arm attached to the base of said nucleotide, said nucleotide being substantially identically located in each of said oligonucleotides, and

wherein said linker arm is further attached to a moiety capable of binding a reporter group or a solid support.

Rivier, 5,807,986

10. A library containing a plurality of betides, each having the formula:

X.sub.N -X.sub.1 -X.sub.2 -X.sub.3 -X.sub.m -X.sub.4 -X.sub.5 -X.sub.6 -X.sub.C, where X.sub.N is an acyl or other N-terminal group or a peptide up to about 50 amino acids in length having such an N-terminal group; X.sub.C is OH, NH.sub.2 or other C-terminal group or a peptide up to about 50 amino acids in length having such a C-terminal group; X.sub.m is either des-X or a peptide up to about 50 amino acids, and X.sub.1 -X.sub.6 are each independently des-X, a betidamino acid, a natural .alpha.-amino acid or an unnatural .alpha.-amino acid,

provided however that at least one of X.sub.1 to X.sub.6 is a residue of a first betidamino acid of the formula: ##STR15## wherein R.sub.0 is H or CH.sub.3, R and R.sub.2 are independently H or substituted or unsubstituted lower alkyl, and R.sub.3 is an acyl group, an isocyanate group, a thioisocyanate group or a sulfonyl group; and that at least another of X.sub.1 to X.sub.6 is either a residue of an .alpha.-amino acid or a residue of a second different betidamino acid of the formula: ##STR16## and provided further however that additional residues of betidamino acids can optionally be included in X.sub.n, X.sub.m and X.sub.c,

each of said betides in said library being formed as a part of a single solid phase peptide synthesis wherein a peptide scaffold containing precursors of said betidamino acids is formed, and wherein a first set of portions of said scaffold are caused to

undergo first addition reactions with reagents to incorporate a plurality of different R.sub.3 groups into said betidamino acid residues of each of said first set of portions.

Peterson 5,783,431

1. A mobilizable combinatorial gene expression library, comprising a pool of expression constructs, each expression construct comprising a shuttle vector that replicates in different species or strains of host cell, said shuttle vector containing one or more cDNA or genomic DNA fragments, wherein the cDNA or genomic DNA fragments in the pool of expression constructs are derived from a plurality of species of donor organisms, and wherein the cDNA or genomic DNA fragments are each operably-associated with one or more regulatory regions that drives expression of genes encoded by the cDNA or genomic DNA fragments in an appropriate host organism.

Lebl 5,840,485

1. A library for identifying and analyzing a ligand of an acceptor of interest comprising: a multiplicity of separate solid phase supports having a plurality of reactive functional groups; to each of which said supports are attached via said functional groups:

a) a species of test compound, said test compound comprised of a sequence of subunits; and

b) one or more species of coding molecule, wherein the coding molecules that are attached to each support:

i) are comprised of .alpha.-amino acids, and

ii) are topologically segregated from the test compound that is attached to each said support, such that the coding molecule is in the interior of each said support, and the test compound is attached to greater than 90% of the reactive functional groups on the exterior of each said support;

wherein, on each said support:

each species of coding molecule is different from the species of test compound; and

the sequence of the subunits of the test compound is encoded by the species of coding molecules.

Kay 5,747,334

1. A library of recombinant vectors in which each vector encodes one of a plurality of heterofunctional fusion proteins comprising:

(a) a binding domain encoded by an oligonucleotide comprising unpredictable nucleotides in which the unpredictable nucleotides are arranged in one or more contiguous sequences, wherein the total number of unpredictable nucleotides is greater than or equal to 60 and less than or equal to 600, wherein the unpredictable nucleotides have the potential to encode all 20 naturally occurring amino acids, and wherein the coding strand of the unpredictable nucleotides comprises the formula (NNB).sub.n+m where

N is A, C, G or T;

B is G, T or C; and

n and m are integers,

such that 20.ltoreq.n+m.ltoreq.200;

wherein the binding domain is encoded by a double stranded oligonucleotide assembled by annealing a first nucleotide sequence of the formula 5' X (NNB).sub.n J Z 3', with a second nucleotide sequence of the formula

3'Z'OU(NNV).sub.m Y5'

at corresponding positions Z and Z',

where X and Y are restriction enzyme recognition sites, such that X.noteq.Y;

N is A, C, G or T;

B is G, T or C;

V is G, A or C;

n is an integer, such that 10.ltoreq.n.ltoreq.100;

m is an integer, such that 10.ltoreq.m.ltoreq.100;

Z and Z' are each a sequence of 6, 9 or 12 nucleotides, such that Z and Z' are complementary to each other; and

J is A, C, G, T or nothing;

O is A, C, G, T or nothing; and

U is G, A, C or nothing; provided, however, if any one of J, O or U is nothing then J, O and U are all nothing; and converting the annealed oligonucleotides to a double stranded oligonucleotide; and

(b) an effector domain encoded by an oligonucleotide sequence encoding a protein or peptide that enhances expression or detection of the binding domain,

said library being capable of being screened to identify a heterofunctional fusion protein having specificity for a ligand of choice.

Kauvar 5,846,722

11. A recombinant host cell in vitro modified to contain:

i) a target protein expression unit which comprises a nucleotide sequence encoding a fusion protein comprising a target protein and a first complementary portion of a segregable active protein; and

ii) a ligand binding domain expression unit which comprises a nucleotide sequence encoding a fusion protein comprising a ligand-binding domain and a second complementary portion of said segregable active protein which is modified to contain an agent/ligand complex comprising a small molecule agent to be tested for binding to said target protein coupled to a ligand which binds said proteinaceous ligand-binding domain.

23. A population of the host cells of claim 11 wherein said population comprises at least two host cells that express target proteins with different amino acid sequences.

24. The population of claim 23 wherein the target proteins are products of expression of a cDNA library.

Kauffman 5,824,514

12. A process for the production of a library of expression vectors capable of producing a transcription product or a translation product, said vectors comprising at least one stochastic sequence of polynucleotides, comprising the steps of:

producing at least one stochastic sequence of polynucleotides;

ligating said stochastic sequence of polynucleotides into an expression vector;

transforming a competent clone with said ligated expression vector;

culturing said transformed clone;

screening and/or selecting said transformed clone in order to isolate a clone expressing a stochastic polynucleotide leading to the synthesis of a transcription product or a translation product;

isolating said selected or screened transformed clone; and

isolating the expression vector cultured in said selected or screened transformed clone so identified.

13. A library of expression vectors capable of producing a transcription product or a translation product, said expression vectors comprising at least one stochastic sequence of polynucleotides, produced in accordance with the process of claim 12.

17. The library of expression vectors according to claim 13 wherein said library comprises stochastic nucleotide sequences encoding for at least 10,000 peptides, polypeptides or proteins.

Kaufmann 5,814,476

1. A process for the production of a transcription product or a translation product, comprising the steps of:

producing a stochastically-generated polynucleotide sequence;

producing a library of expression vectors comprising said stochastic polynucleotide sequence;

transforming or transfecting a competent clone with said library of expression vectors;

amplifying said transformed or transfected competent clone;

screening and/or selecting said transformed or transfected clone in order to isolate a clone expressing a stochastic polynucleotide sequence capable of synthesizing a transcription

product or a translation product having a predetermined property; and

isolating said selected or screened transformed clone;

isolating a stochastically generated polynucleotide sequence which encodes the identified transcription product or translation product

using the isolated sequence to produce the transcription product or translation product having the predetermined property.

14. A library of vectors produced by the process of claim 1 wherein said library comprises stochastic nucleotide sequences encoding for at least 10,000 transcription products or translation products.

Houston 5,824,483

1. A combinatorial library of different-sequence polypeptide members, where each member of the library comprises

(a) first and second polypeptides bound to one another to form an alpha-helical coiled-coil dimer scaffold characterized by (i) an internal region formed by regularly repeating, invariant, hydrophobic amino acid residues in both polypeptides, (ii) first and second exposed regions formed by regularly repeating amino acid residues in the individual first and second polypeptides, respectively, (iii) a polypeptide length of at least seven residues for each polypeptide, and (iv) a covalent intrachain bond between invariant residues in at least one of the polypeptides, effective to stabilize that polypeptide in its .alpha.-helical conformation, where the scaffold is stabilized by hydrophobic interactions among the subunits in the internal region of the scaffold and the intrachain bond, and

(b) a unique variation of amino acid residues in the exposed region of at least one of the polypeptides.

Houghten 5,846,731

1. A set of linear peralkylated oligopeptide chains comprising a mixture of equimolar amounts of linear peralkylated oligopeptide chain members containing the same number of about two to about ten peralkylated amino acid residues in each chain, each peralkylated amino acid residue except proline having its Deptidyl amido nitrogen atom alkylated with a C.sub.1 -C.sub.7

alkyl group, the members of said set having one or more of at least six different peralkylated amino acid residues at the same one or more predetermined positions of the peralkylated oligopeptide chain, and the set having equimolar amounts of at least six different of said peralkylated amino acid residues at one or more of the same other positions of the peralkylated oligopeptide chain, the amino-terminus of each peralkylated oligopeptide being selected from the group consisting of a quaternary alkylammonium group, an amino group, an N-alkylamino, an N-alkyl-N-C.sub.1 -C.sub.18 hydrocarboyl and a pyroglutamoyl group, and the carboxy-terminus being selected from the group consisting of an alkyl carboxylic ester, mono- or di-N-alkylcarboxamide and a carboxyl group.

Holmes 5,770,456

1. An array of cyclic nucleic acids on a substrate, said nucleic acids having N nucleotide positions, said substrate comprising N different sites, said substrate sites comprising said cyclic nucleic acids coupled thereto, said cyclic nucleic acids comprising common nucleotide sequences but coupled to said substrate at a different one of said nucleotide positions via a tether molecule in each of said different substrate sites.

5. An array of cyclic polypeptides on a substrate, said polypeptides having N amino acid positions, said substrate comprising N different sites, said substrate sites comprising said cyclic polypeptides coupled thereto, said cyclic polypeptides comprising common amino acid sequences but coupled to said substrate at a different one of said amino acid positions via a tether molecule in each of said different substrate sites.

Hodges 5,738,996

1. For use in selecting an oligomer compound capable of interacting specifically with a selected macromolecular ligand, a combinatorial library composition comprising

a first set of combinatorial oligomer libraries in which one or more selected subunit positions in the library oligomers have one of substantially all possible different subunits in each of the selected positions, and the remaining one or more subunit positions in each library include substantially all possible combinations of the different subunits, and

a second set of combinatorial oligomer libraries in which one or more different selected subunit positions in the library oligomers have one of substantially all possible different

subunits in each of the selected positions, and the remaining one or more subunit positions in each library include substantially all possible combinations of the different subunits,

wherein the subunits are representative amino acids that display the basic physico-chemical properties associated with naturally occurring amino acids, but exclude many of these naturally occurring amino acids.

Hamilton, 5,770,380

1. An antibody mimic comprising a calixarene organic scaffold to which a plurality of peptide loops are covalently linked.

Dower, 5,770,358

1. An encoded library of compounds comprising:

a solid support;

a plurality of copies of a compound directly bound to said support, said compound having a first reactive group selected from the group consisting of amino groups, carboxyl, hydroxyl, and phosphate groups and wherein any additional reactive groups of said compound that are capable of interfering with compound synthesis or identifier tag addition have been suitably protected;

one or more first identifier tags, each tag being directly bound to said support, said tag having a second reactive group selected from the group consisting of amino groups, carboxyl, hydroxyl, and phosphate groups and wherein any additional reactive groups of said tag that are capable of interfering with compound synthesis or identifier tag addition have been suitably protected, wherein said identifier tag is different than said compound, and

wherein said tag identifies the compound bound to said support or records a step in a synthesis of said compound; and

a substance in contact with said support selected from the group consisting of a building block having a third reactive group, a second identifier tag having a fourth reactive group, an activator group, and a solvent, wherein said third reactive group of said building block and said fourth reactive group of said

second identifier tag are independently selected from the group consisting of amino groups, carboxyl, hydroxyl, and phosphate groups and wherein any additional reactive groups of said building block or said second identifier tag that are capable of interfering with compound synthesis or identifier tag addition have been suitably protected.

Cook 5,831,014

1. A compound of the structure:

>AA!.sub.w --{>PNA!.sub.u -->AA!.sub.v }.sub.x -->PNA!.sub.y -->AA!.sub.z

wherein

each AA, independently, is an amino acid residue;

each PNA, independently, is a peptide amino acid residue;

u, v, x and y, independently, are 1 to 500;

w is 0 to 500;

z is 3 to 500; and

the sum of u, v, w, x, y and z is less than 500.

Sanghvi 5,808,023

1. A compound having the structure: ##STR4## wherein: Z, is C(O)H, CH.sub.2 C(O)H, CH.sub.2 R.sub.A NH.sub.2, or R.sub.A NH.sub.2 ;

R.sub.A is O or NR.sub.1 ;

Y.sub.1 is hydroxymethyl, a nucleoside attached via a linking group, a nucleotide, an oligonucleotide, an oligonucleoside, or a hydroxyl-protected or amine-protected derivative thereof;

R.sub.1 is H; alkyl or substituted alkyl having 1 to about 10 carbon atoms; alkenyl or substituted alkenyl having 2 to about 10 carbon atoms; alkynyl or substituted alkynyl having 2 to about 10 carbon atoms; alkaryl, substituted alkaryl, aralkyl, or substituted aralkyl having 7 to about 14 carbon atoms;

B.sub.X1 is a nucleosidic base;

Q.sub.1 is O, S, or CH.sub.2 ; and

X.sub.1 is H; OH; alkyl or substituted alkyl having 1 to about 10 carbon atoms; alkaryl, substituted alkaryl, aralkyl, or substituted aralkyl having 7 to about 14 carbon atoms; F; Cl; Br; CN; CF.sub.3 ; OCF.sub.3 ; OCN; O-alkyl; S-alkyl; N-alkyl; O-alkenyl; S-alkenyl; N-alkenyl; SOCH.sub.3 ; SO.sub.2 C.sub.3 ; ONO.sub.2 ; NO.sub.2 ; N.sub.3, NH.sub.2 ; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; or polyalkylamino or substituted silyl.

Still US 6,001,579

1. A solid support comprising:

a) a compound, said compound having been synthesized by a reaction series comprising a first stage reagent and a first stage reaction condition and a second stage reagent and a second stage reaction condition, wherein the first stage precedes the second stage in the reaction series;

b) a first plurality of identifiers each having a non-sequencable tag, the combination of which tags records the first stage reagent or the first stage reaction condition; and

c) a second plurality of identifiers each having a non-sequencable tag, the combination of which tags records the second stage reagent or stage second reaction condition,

wherein the tags of the first and the second pluralities are different each from the other, and wherein each identifier is attached to the solid support through other than the compound.

13. A library comprising at least 100 solid supports according to claim 1, that are unique compared to each other.

Rebek, Jr., 5,877,030

1. A method for forming a combinatorial library, the method comprising:

(a) admixing a plurality of xanthene molecules with a mixture of amino acids to form a reaction mixture, wherein each of said xanthene molecules has from two to four reactive centers attached thereto, each of said reactive centers is independently selected from the group consisting of an acid halide, epoxide, aldehyde, carboxylic acid, and carboxylic ester, and each of said amino acids has an amine group that is capable of reacting with said reactive centers;

(b) reacting the reactive centers of the xanthene molecules with the amine groups of the amino acids to form a mixture of library molecules.

9. A combinatorial library produced by the process of claim 1.

Mandecki 6,001,571

11. A method of detecting target nucleic acids in a sample, comprising the steps of:

(a) introducing into the sample at least two populations of solid phase particles, each particle having a transponder and having an oligonucleotide probe attached to its surface, a first population having an oligonucleotide probe that hybridizes to a different target nucleic acid than a second population and the transponders in the first population being encoded with a different identification than the transponders of the second population;

(b) denaturing the nucleic acids in the sample;

(c) hybridizing the target nucleic acids to the oligonucleotide probes;

(d) analyzing the particles to detect a label indicating that target nucleic acid has bound to the probe; and

(e) decoding the transponder to identify the probe.

12. The solid phase of claim 11, wherein the solid phase comprises at least three populations of solid phase particles, each particle having a transponder and having an oligonucleotide probe attached to its surface, each of the three populations having a different oligonucleotide probe sequence and each of the populations being encoded with a different. identification than the transponders of the second population.

Kang 5,955,341

1. A phagemid vector for expressing first and second fusion proteins that form a phagemid-anchored heterodimer upon expression in a host, said vector comprising:

a) a first fusion protein expression cassette comprising (i) a first prokaryotic secretion signal-encoding sequence operatively linked upstream via a first directional ligation sequence to an Ff filamentous phage gene cpVIII membrane anchor-encoding sequence, and (ii) a first set of DNA expression signals including a promoter and a ribosome binding site operatively linked upstream to said first prokaryotic secretion signal-encoding sequence and at least one stop codon in frame with said membrane anchor-encoding sequence; and

b) a second fusion protein expression cassette comprising (i) a second prokaryotic secretion signal-encoding sequence operatively linked upstream to a second directional ligation sequence, and (ii) a second set of DNA expression signals including a promoter and a ribosome binding site operatively linked upstream to said second prokaryotic secretion signal-encoding sequence.

9. A library of Ff filamentous phage wherein said phage contains a DNA expression vector according to claim 1.

Huse 5,871,974

1. A composition of matter comprising a plurality of cells containing only operatively combined vectors having diverse combinations of first and second DNA sequences encoding first and second polypeptides which form heteromeric receptors of the immunoglobulin superfamily, one or both of said polypeptides being expressed as fusion proteins on the surface of a cell or a bacteriophage, said operatively combined vectors being formed by the recombination of first and second vectors having two p airs of restriction sites symmetrically oriented about a cloning site for containing said first and second DNA sequences, said recombination effected between the two pairs of restriction sites.

Geysen 5,998,577

1. A set of catamers, comprising:

a plurality of catamers having the general formula:

Y-X.sub.1 -X.sub.2 -X.sub.3 -X.sub.4 -(X).sub.n -S

wherein one of X is a designated monomer and each remaining X is independently a monomer randomly selected from a set of monomers, said set of monomers being selected from the group consisting of L-amino acids and D-amino acids;

Y is selected from the group consisting of H, acetyl and protecting groups;

S is a solid support; and

n is an integer from 0 to 4.

Fowlkes 5,876,951

29. A mixture of recombinant yeast cells, each cell of which comprises:

(i) a pheromone system generating a detectable signal;

(ii) an expressible gene construct encoding a heterologous surrogate of a yeast pheromone system protein, said surrogate being a farnesyl transferase and performing in the pheromone system; and

(iii) an expressible gene construct encoding a heterologous peptide, said heterologous peptide including a signal sequence for secretion into the periplasmic space,

wherein collectively the mixture of cells express a library of said heterologous peptides, and modulation of the pheromone system by the heterologous peptide provides the detectable signal.

Cook 5,864,010

1. A compound of the structure:

›AA!.sub.w -{›PNA!.sub.u ->AA!.sub.v }.sub.x ->PNA!.sub.y ->AA!.sub.z

wherein;

each AA, independently, is an amino acid residue;

each PNA, independently, is a peptide nucleic acid residue which comprises a C.sub.2 -C.sub.6 alkyldiamine moiety in the backbone;

u, v, x and y, independently, are 1 to 500;

w and z, independently, are 0 to 500;

provided that when w is 0, then z is greater than 1; and

the sum of u, v, w, x, y and z is less than 500.

Boger 5,939,268

1. A mixture library comprising a mixture of compounds formed by reaction of one of a first through nth diene and one of a first through nth dienophile one through n times utilizing the Diels-Alder reaction, wherein n is 2 to 500.

Lerner 6,060,596

1. A bifunctional molecule according to the formula A--B--C, wherein A is a polymer comprising a linear series of chemical units represented by the formula (X.sub.n).sub.a, wherein X is a single chemical unit in polymer A, B is a linker molecule operatively linked to A and C. and identifier oligonucleotide C is represented by the formula (Z.sub.n).sub.a, wherein a unit identifier nucleotide sequence Z within oligonucleotide C identifies the chemical unit X at position n; and

wherein n is a position identifier for both X in polymer A and Z in oligonucleotide C having the value of 1+i where i is an integer from 0 to 10, such that when n is 1, X or Z is located most proximal to the linker, and a is an integer from 4 to 50.

10. A library comprising a plurality of species of bifunctional molecules according to claim 1.

11. The library of claim 10 wherein said plurality of species is defined by the formula V.sup.a, where V represents the number of different chemical units forming an alphabet of possible chemical units of X, and a is an exponent to V and represents the number of chemical units of X forming polymer A.

12. The library of claim 11 wherein X is a natural amino acid and V is 20.

Lebl 6,090,912

1. A library for identifying a ligand or an acceptor of interest, the library comprising a multiplicity of separate solid phase supports, the surface of each support having attached a linker comprising a single species of test compound having a sequence of subunits, and the interior of each support having attached a coding molecule which encodes the sequence of subunits of the test compound, the linker having a bond that is cleavable by an enzyme that does not cleave a bond of the coding molecule.

2. The library of claim 1, wherein the linker is a peptide.

Cook 6,756,199

1. A combinatorial library of compounds having the structure:

(AA)w-{(PNA)u-(AA)v}x-(PNA)y-(AA)z

wherein

each AA, independently, is an .alpha.- or .beta.-amino acid residue;

each PNA, independently, is a peptide nucleic acid residue having an aminoethylglycine backbone and a nucleobase bound thereto directly or through a tether,

u, v, x and y are each independently 1 or greater than 1;

w and z, independently, are 0 to 500; and

the sum of u, v, w, x, y and z is less than 500;

wherein said AA and said PNA residues are linked directly together by amide linkages.

Anderson 6,562,617

1. A library of fusion nucleic acids each comprising:

a) a first nucleic acid encoding a Renilla GFP scaffold protein;

b) a second nucleic acid encoding a different random peptide fused to the N-terminus of said scaffold protein; and

c) a third nucleic acid encoding a flexible linker between said scaffold and said random peptide.

Appendix 2: Combinatorial Library Patents With Broad Method of Making or Method of Use Claims (partial list)

Houghten 4,631,211 (1986)

1. A means for carrying out a sequential, solid phase organic synthesis to form a reaction product containing a plurality of reacted subunits comprising an immersible mesh packet having enclosed therein a free form aliquot of reactive particles, said particles being of a size that is larger than any of the foraminae defined by said mesh packet and having a known amount of organic synthesis reactive functionality covalently linked to said particles, said reactive functionality being capable of reacting during said organic synthesis, said mesh packet and said particles being substantially insoluble in water and in organic solvents.

Kauvar 4,963,263 (1990)

1. A method to identify a peptide having a sequence of 4-20 amino acids useful for the conduct of affinity chromatography with respect to an analyte,

wherein said peptide has specific affinity for said analyte, which method comprises:

screening, for ability to bind said analyte, a panel of individual candidate peptides wherein said candidate peptides have systematically varied values of at least two parameters selected from the group consisting of hydrophobic index, amphipathic characteristics, and charge pattern.

Rutter 5,010,175 (1991)

1. A method of preparing in the same reaction vessel a mixture of peptides of distinct, unique and different sequences which mixture contains each peptide in retrievable and analyzable amounts and in substantially equal molar amounts, comprising:

combining and reacting activated amino acids with an acceptor amino acid or peptide wherein said activated amino acids are provided in concentrations relative to each other based on the relative coupling constants so that the mixture of the peptides resulting from the reaction contains each of the peptides in predictable and defined amounts sufficient for each of the peptides to be retrieved and analyzed.

9. A method of obtaining a peptide or mixture of peptides of a

specified target property, which method comprises the steps of:

providing in the same vessel a mixture of candidate peptides which mixture contains at least about fifty candidate peptides with each of the candidate peptides being present in retrievable and analyzable amounts and in substantially equal molar amounts; and

selecting from among the candidate peptides one or more peptides having a desired target property and separating such away from those not having the target property.

Pirrung 5,143,854

1. A method of identifying at least one polypeptide for binding with a receptor comprising the sequential steps of:

a) generating a pattern of light and dark areas by selectively irradiating at least a first area of a surface of a substrate, said surface comprising polypeptides immobilized on said surface, said polypeptides having a photoremovable protective group, without irradiating at least a second area of said surface, to remove said protective group from said polypeptides in said first area;

b) simultaneously contacting said first area and said second area of said surface with a first amino acid to couple said first amino acid to said polypeptides in said first area, and not in said second area, said first amino acid having said photoremovable protective group;

c) generating another generating another pattern of light and dark areas by selectively irradiating with light at least a part of said first area of said surface and at least a part of said second area to remove said protective group in said at least a part of said first area and said at least a part of said second area;

d) simultaneously contacting said first area and said second area of said surface with a second amino acid to couple said second amino acid to said polypeptides in said at least a part of said first area and said at least a part of said second area;

e) performing additional irradiating and amino acid contacting and coupling steps so that a matrix array of at least 100 different polypeptides is formed on said surface, each different polypeptide synthesized in an area of less than 0.1 cm.sup.2, whereby said different polypeptides have amino acid sequences and

locations on said surface defined by the patterns of light and dark areas formed during the irradiating steps and the amino acids coupled in said contacting steps; and

f) contacting said surface with a receptor and identifying which polypeptides on said surface specifically bind to said receptor.

Kauvar 5,133,866

1. A method to identify a paralog useful for the conduct of affinity chromatography with respect to an analyte which has specific affinity for a first moiety in comparison to additional moieties present in the environment of the first moiety which method comprises:

screening, for ability to selectively bind said first moiety a panel of individual candidate paralogs, wherein said candidate paralogs have systematically varied values of at least two different parameters, each of which parameters determines the ability of the paralog to bind other substances.

Beattie, 5,175,209

30. A process for simultaneous chemical synthesis of a multiplicity of biopolymer sequences, comprising the steps of:

assembly of modular chemically inert wafers in abuttably stacked relationship to form a multiplicity of wafer columns;

sequentially passing reagents through wafer columns to effect addition of a different biopolymer residue to growing biopolymers contained within each column;

reassortment of wafers into a new abuttably stacked relationship to provide for synthesis of a multiplicity of biopolymer sequences;

repetition of residue addition steps comprising assembly of columns, biopolymer residue addition, and reassortment of wafers, until synthesis of the desired multiplicity of biopolymer sequences has been achieved.

Schatz 5,270,170

1. A method for screening a random peptide library to identify a plasmid that encodes a peptide that binds to a receptor, said

library comprising at least 10.sup.6 different transformed E. coli host cells, each cell containing a plasmid that comprises a lacO binding site and encodes a fusion protein comprising a lac repressor DNA binding protein fused to a peptide, wherein said 10.sup.6 different transformed host cells each differ from one another with respect to the peptide in said fusion protein, said method comprising the steps of:

(a) lysing the transformed host cells under conditions such that the fusion protein remains bound to the vector plasmid that encodes the fusion protein;

(b) contacting the fusion proteins of the peptide library with a receptor under conditions conducive to specific peptide--receptor binding; and

(c) isolating the plasmid that encodes a peptide that binds to said receptor.

Rutter 5,225,533

1. A method of preparing a mixture of distinct, unique and different peptides in the same reaction vessel, which mixture contains each peptide in retrievable and analyzable amounts, comprising:

combining and reacting activated amino acids with an acceptor amino acid or peptide wherein said activated amino acids are provided in concentrations relative to each other based on the relative coupling constants so that the mixture of the peptides resulting from the reaction contains each of the peptides in predictable and defined amounts sufficient for each of the peptides to be retrieved and analyzed.

Ladner 5,223,409

1. A method of obtaining a nucleic acid encoding a binding protein having a proteinaceous binding domain that binds a predetermined target material, said target being a substance other than an antibody with an exposed antigen-combining site, comprising:

a) preparing a variegated population of amplifiable genetic packages, said genetic packages being selected from the group consisting of cells, spores and viruses, each said genetic package being genetically alterable and having an outer surface including a genetically determined outer surface protein, each package including a first nucleic acid construct coding for a

chimeric potential binding protein, each said chimeric protein comprising and each said construct comprising DNA encoding (i) a potential binding domain which is a mutant of a predetermined domain of a predetermined parental protein other than a single chain antibody, and (ii) an outer surface transport signal for obtaining the display of the potential binding domain on the outer surface of the genetic package, the expression of which construct results in the display of said chimeric potential binding protein and its potential binding domain on the outer surface of said genetic package: and wherein said variegated population of genetic packages collectively display a plurality of different potential binding domains, the differentiation among said plurality of different potential binding domains occurring through the at least partially random variation of one or more predetermined amino acid positions of said parental binding domain to randomly obtain at each said position an amino acid belonging to a predetermined set of two or more amino acids, the amino acids of said set occurring at said position in statistically predetermined expected proportions, said genetic packages being amplifiable in cell culture and separable on the basis of the potential binding domain displayed thereon,

b) causing the expression of said chimeric potential binding proteins and the display of said potential binding domains on the outer surface of said packages;

c) contacting said packages with the predetermined target material such that said potential binding domains and the target material may interact;

d) separating packages displaying a potential binding domain that binds the target material from packages that do not so bind, and

e) recovering at least one package displaying on its outer surface a chimeric binding protein comprising a successful binding domain (SBD) which bound said target, said package comprising nucleic acid encoding said successful binding domain, and amplifying said SBD-encoding nucleic acid in vivo or in vitro.

Huebner 5,182,366

1. A method of preparing a mixture of peptides having different amino acid sequences, which mixture contains retrievable and analyzable amounts of each peptide, comprising:

(a) dividing an amount of a mixture of amino acyl or peptide derivatized resins into a plurality of subamounts;

(b) coupling a different single amino acid with each of the subamounts of resin to obtain a plurality of different peptide resins the coupling being carried out under conditions such that it is driven to substantial completion with each subamount; and

(c) combining known amounts of the different peptide resins together to obtain the mixture of peptides; which mixture contains retrievable and analyzable amounts of each of the peptide resins.

Geysen 5,194,392
1. A method of determining the sequences of amino acids which is a conformational equivalent of an epitope which is complementary to a particular paratope of an antibody of interest, the method comprising the steps of:

a. synthesizing a plurality of catamer preparations; each catamer having at least four positions; each of said catamer preparations consisting of a plurality of catamers made of a defined set of at least four amino acids and in which:

(i) a residue at two or more designated positions in each catamer are known and are constant for each catamer in the catamer preparation ("constant positions"),

(ii) the residue at each of the remaining positions is randomly made up from members selected from said defined set of amino acids ("random positions"); wherein at least two of the positions of said catamer are random; and

(iii) said plurality of catamer preparations comprising preparations in which the residue at the designated positions is systematically varied to contain members from said defined set of amino acids, wherein the number of catamers in said catamer preparation is the product of the number of amino acids selected for each random position;

b. contacting each of said plurality of catamer preparations with the antibody of interest;

c. detecting the presence or absence of binding between each of said plurality of catamer preparations and said given antibody;

d. determining a partial sequence of amino acids for the conformational equivalent of an epitope which is complementary to the particular paratope of said antibody from the known residue at two or more designated positions in each of said plurality of catamer preparations that bind to said antibody;

e. synthesizing a further plurality of catamer preparations as defined in step a and which include the composition of the partial sequence as determined in said step d as initial designation positions, each of said further plurality of catamer preparations having at least one additional position in each catamer as a designated position; and

f. repeating steps b, c and d with said further plurality of catamer preparations.

lebl 5,202,418

1. A method of synthesizing peptides, comprising the steps of:

providing a planar carrier on a disk, which carrier is divided into a plurality of individual compartments, each compartment containing an inert porous material, a circular path being defined around said disk, said compartments being circumferentially spaced along said circular path;

anchoring at least one functional group for synthesis of at least one peptide onto the inert porous material of each compartment to form a plurality of individual functionalized compartments;

arranging a dosing head at a fixed location adjacent said circular path, the dosing head including means for directly applying measured quantities of at least one liquid component from a common reservoir of such component;

positioning the disk so that one of the individual functionalized compartments is positioned to receive a liquid component directly applied by the closing head;

directly applying a measured quantity of a liquid component to an individual functionalized compartment from the common reservoir thereof, via the dosing head, the applied component providing an amino acid to form a covalent bond with the functional group of the individual functionalized compartment positioned to receive the liquid component for beginning the synthesis of a peptide from the functional group of the individual functionalized compartment receiving the measured quantity;

rotating the disk to position another individual compartment along the circular path to receive a liquid component applied by the dosing head; and

subsequently applying at least one other amino acid to another individual functionalized compartment in at least one other step

for completing the synthesis of peptides.

Anderson 5,272,075

1. A process for synthesizing a polymer selected from the group consisting of an oligonucleotide, a peptide, a polysaccharide and a heteropolymer comprising two or more elements of an oligonucleotide, polypeptide, polysaccharide, intercalating agent, enzyme or cytoxic agent, said process comprising introducing two series of solutions into a rotating rotor body containing a solid support matrix having a seed monomer wherein one series of solutions of an increasing density is introduced at the rotor edge and the second series of solutions of a decreasing density is introduced at the rotor center for the addition of a monomer to said seed monomer, repeating the introduction of the two series until the desired polymer is obtained, and recovering the synthesized polymer.

Kauvar 5,338,659

1. A method to identify analyte and to determine analyte concentration of a sample containing at least one member analyte of a set of analytes cross-reactive with at least two specific binding reagents, wherein said cross-reactive set of analytes contains at least 2 members, which method comprises:

1) contacting the sample with each specific binding reagent of a panel containing n specific binding reagents reactive with the members of the set wherein n is an integer and is at least 2;

2) measuring the binding affinity of each specific binding reagent in the panel with the sample;

3) recording each said binding affinity of each said binding reagent in said panel;

4) arranging said recorded binding affinities into a convenient pattern for comparison to obtain a cross-reactivity of specific binding reagents (CRSBR) profile of said sample; and

5) comparing said CRSBR profile thus obtained with a set of CRSBR reference profiles for individual member analytes and mixtures of member analytes thereby to find a matching CRSBR reference profile corresponding to a composition for which the identity and concentration of each said member analyte is known,

wherein each CRSBR reference profile for an individual member

analyte consists of binding affinities for all specific binding reagents of said panel against a single concentration of an individual member analyte arranged in said convenient pattern in a physical embodiment, and

wherein each CRSBR reference profile for a mixture of said member analytes consists of binding affinities for all specific binding reagents of said panel against a single concentration of a single mixture of two or more member analytes arranged in said convenient pattern, said binding affinities for said single mixture being calculated by combining said recorded binding affinities for single concentrations of said individual member analytes.

Kauvar 5,300,425

1. A method to select a successful member from a panel of candidate drugs, said successful member being capable of binding to a known receptor, which method comprises:

(a) providing a profile of reactivity of said receptor against a panel of maximally diverse mimotopes;

(b) providing a panel of antibodies which is an inverse image of said maximally diverse panel of mimotopes in that each antibody binds to one of said mimotopes with a binding constant at least twenty times greater than that with any of the other mimotopes in the panel;

(c) preparing a profile of the binding of the candidate drug to the members of the inverse image antibody panel;

(d) matching the mimotype-binding profile of the receptor with the inverse image profile of the candidate drug, and

(e) selecting as said successful member a candidate drug which has an inverse image profile which is similar to the mimotype-binding profile of the receptor.

Pirrung 5,405,783

1. A method of forming an array of diverse peptides on a substrate, the surface of said substrate comprising at least first and second regions having peptide molecules thereon, said peptide molecules coupled to a photoremovable protective group at a first functional group, said first functional group capable of binding to a second functional group of selected amino acids which also have a first functional group coupled to a

photoremovable protective group, said method comprising the steps of:

removing said photoremovable protective group from said peptide molecules in said first region of said substrate with light to expose said first functional group, but not removing said photoremovable protective group from said peptide molecules in said second region;

contacting said first and second regions of said surface with first selected amino acid molecules to covalently bond said second functional group of said first amino acid molecules to said first functional group of said peptides by means of a peptide bond in said first region, but not said second region;

removing the photoremovable protective group from at least a portion of said first selected amino acid molecules in said first region with light to expose said first functional group on said at least a portion of said first selected amino acid molecules; and

contacting said first and said second regions with second selected amino acid molecules to covalently bond a second functional group of said second selected amino acid molecules to said first functional group of said first selected amino acid molecules by means of a peptide bond, forming peptides in said first region having a different amino acid sequence than peptides in said second region.

Nishioka 5,449,754

1. A method of making chemical compounds of preselected varying sequenced chemical units attached to the surface of a solid support comprising:

a) injecting droplets of a first liquid solution containing one of said units that is disposed to attach to said surface from nozzles constructed in the manner of an ink-jet printing head positioned to deposit said droplets onto said surface in separate selective locations on said surface so that such units attach to the surface of said support; and

b) injecting droplets of a second liquid solution containing one of said units that is disposed to attach to the units of said first liquid coupling solution from said nozzles positioned to cause said second droplets to impinge onto the selective locations so that the units of said second liquid attach to the units of said first liquid coupling solution attached to said

support.

Matson 5,429,807

11. A method for synthesizing biopolymer comprising the steps of:

providing a fixed polypropylene support having a surface activated for attaching biopolymers;

providing an applicator in contact with said activated surface for applying reagents to said activated surface;

positioning the applicator with respect to the activated surface and applying reagents to said activated surface to synthesize a first one-dimensional array of biopolymers;

repositioning the applicator and applying reagents to said activated surface to synthesize a second one-dimensional array of biopolymers in a manner where said second one-dimensional array overlaps the first one-dimensional array to form a two-dimensional array of cells at overlapping regions on the activated surface in each of which a third biopolymer is obtained which includes a corresponding biopolymer from said first and second arrays of biopolymers.

Kauvar 5,409,611

1. A method to characterize a single analyte, which method comprises:

contacting said analyte with each of a panel of diverse paralogs which react in a multiplicity of differing degrees with said single analyte;

detecting the degree of reactivity of said analyte to each of said paralogs;

recording said degree of reactivity of said analyte to each of said paralogs; and

arranging said recorded degrees of reactivity so as to provide a characteristic profile of said analyte.

Fodor 5,424,186

1. A method for synthesizing oligonucleotides on a solid phase comprising the steps of:

a) providing a substrate as the solid phase, wherein said substrate comprises oligonucleotide molecules immobilized on a surface thereof, said oligonucleotide molecules coupled to a photoremovable protecting group;

b) irradiating a first predefined region of said substrate without irradiating a second predefined region of said substrate to remove said protecting group from said oligonucleotide molecules in said first region; and

c) contacting said substrate with a first nucleotide to couple said first nucleotide to said oligonucleotide molecules in said first predefined region, said first nucleotide having a nucleotide protecting group thereon, forming a first oligonucleotide on said substrate in said first predefined region that is different from an oligonucleotide in said second predefined region.

Dower 5,432,018

3. A method for identifying a peptide which binds to a preselected receptor and measuring a dissociation rate for the binding of said peptide to said receptor, comprising; p1 (a) transforming host cells with at least 10.sup.6 different bacteriophage expression vector wherein each of said vectors encodes a fusion protein composed of a peptide fused to a coat protein of a filamentous bacteriophage, and wherein said vectors differ from each other with respect to the peptide of said fusion protein encoded by said vector;

(b) cultivating said transformed host cells under conditions suitable for expression and assembly of bacteriophage;

(c) contacting bacteriophage displaying the peptide to the preselected receptor under conditions conducive to specific peptide-receptor binding;

(d) selecting bacteriophage which bind to the receptor;

(e) separating bound bacteriophage selected in step (d) into individual isolates;

(f) binding each isolate separated in step (e) to a labeled monovalent receptor; and

(g) measuring over time how much receptor binds each isolate in the presence and absence of a known ligand for said receptor to determine a dissociation rate for the binding of said peptide to

said receptor.

Pinilla 5,556,762

1. A process for providing the amino acid residue sequence of an oligopeptide ligand that specifically binds to an acceptor that comprises the steps of:

(a) providing separate pluralities of sets of self-solubilizing, unsupported mixed oligopeptides, each of said pluralities having sets that consist essentially of a mixture of equimolar amounts of linear oligopeptide chains containing five to about ten amino acid residues in each chain, the members of each set having one of at least six different predetermined amino acid residues at a single, predetermined position of the oligopeptide chain, and having said at least six different amino acid residues at the same other positions of the oligopeptide chain, each set having equimolar amounts of said at least six different amino acid residues at said other positions in the oligopeptide chain, but differing from the other sets in that the identity and chain position of the one of at least six predetermined amino acid residues present at the predetermined chain position within each set is different between the sets, each plurality of sets differing from the other plurality of sets by the chain position of said one of at least six different predetermined amino acid residues, the number of sets in said separate pluralities of sets being equal to the product of the number of different amino acid residues present at said predetermined chain positions containing said one of at least six different residues times the number of different chain positions containing said one of at least six predetermined amino acid residues;

(b) separately admixing each set with said acceptor in an aqueous medium at a set concentration of about 0.1 milligrams per liter to about 100 grams per liter, separately assaying the binding of each set to said acceptor, and determining the one or more sets that provided specific binding for each different chain position,

the identity and position of the amino acid residue of said each one or more sets that provided specific binding for each chain position providing the amino acid residue sequence for the ligand that specifically binds to said acceptor.

2. The process according to claim 1 wherein the positions of the oligopeptides in the set pluralities having equimolar amounts of different amino acid residues are occupied by about 15 to about 20 different amino acid residues.

Lam 5,510,240

5. A method of screening a peptide library and determining a sequence of a peptide ligand for an acceptor molecule of interest comprising the steps of:

a) introducing an acceptor molecule to a peptide library, wherein the library is comprised of a multiplicity of solid phase supports having a linker, wherein a single peptide species is covalently attached by the linker to each solid phase support, and in which the library is prepared by a method comprising the steps of:

(i) providing at least two aliquots of solid phase supports, said solid phase supports having an N.sup..alpha. reactive site;

(ii) separately introducing a species of subunit of the peptide to each of the aliquots of solid phase supports such that a different species of subunit is introduced into each aliquot, said subunits having a protecting group or a plurality of protecting groups;

(iii) completely coupling the subunit to substantially all the reactive sites of the solid phase supports;

(iv) thoroughly mixing and deprotecting the aliquots of solid phase supports whereby an N.sup..alpha. reactive site is provided; and

after completing all desired executions of steps (i) through (iv), a final step of removing any remaining protecting groups such that the peptides remain covalently attached to the solid phase supports;

b) identifying the solid-phase supports having peptides thereon that bind the acceptor molecule, wherein said identified supports represent at most about 1 part per 1,000 parts of the supports of the library;

c) isolating at least one identified support from all other species of the library; and thereafter

d) determining the sequence of the peptide species attached to the isolated support.

Ladner 5,571,698

1. A method of obtaining a nucleic acid encoding a binding

protein having a proteinaceous binding domain that binds a predetermined target material comprising:

a) preparing a variegated population of amplifiable genetic packages, said genetic packages being selected from the group consisting of cells, spores and viruses, each said genetic package being genetically alterable and having an outer surface including a genetically determined outer surface protein, each package including a first nucleic acid construct coding for a chimeric potential binding protein, each said chimeric protein comprising and each said construct comprising nucleic acid encoding (i) a potential binding domain which is a mutant of a predetermined domain of a predetermined parental protein other than a single chain antibody, and (ii) an outer surface transport signal for obtaining the display of the potential binding domain on the outer surface of the genetic package, the expression of which construct results in the display of said chimeric potential binding protein and its potential binding domain on the outer surface of said genetic package; and wherein said variegated population of genetic packages collectively display a plurality of different potential binding domains, the differentiation among said plurality of different potential binding domains occurring through the at least partially random variation of one or more predetermined amino acid positions of said parental binding domain to randomly obtain at each said position an amino acid belonging to a predetermined set of two or more amino acids; the amino acids of said set occurring at said position in statistically predetermined expected proportions, said genetic packages being amplifiable in cell culture and separable on the basis of the potential binding domain displayed thereon,

b) causing the expression of said chimeric potential binding proteins and the display of said potential binding domains on the outer surface of said packages;

c) contacting said packages with the predetermined target material such that said potential binding domains and the target material may interact;

d) separating packages displaying a potential binding domain that binds the target material from packages that do not so bind, and

e) recovering at least one package displaying on its outer surface a chimeric binding protein comprising a successful binding domain (SBD) which bound said target, said package comprising nucleic acid encoding said successful binding domain, and amplifying said SBD-encoding nucleic acid in vivo or in vitro,

with the proviso that when the target is an antibody, the predetermined parental protein is not an antigen specifically bound by that antibody.

Blake 5,565,325

1. A method for identifying a peptide which binds to a ligand on interest, comprising:

(a) synthesizing a first library of random or semi-random soluble peptides of from three to eight amino acids in length and screening the library for binding to the ligand of interest to establish a baseline binding activity;

(b) synthesizing a second soluble peptide library wherein at a single residue position of the peptide library at least two different groups of amino acids are used to prepare the library, in which the groups are each added at said residue position at different molar concentrations, and wherein the number of amino acids in each group is less than the number of amino acids used at said residue position in the synthesis of the library of step (a);

(c) screening the second peptide library for binding to the ligand of interest and comparing the binding activity thereof to the baseline activity of the first library, thereby identifying the group of amino acids which contributes to optimal binding to the ligand of interest;

(d) synthesizing a third soluble peptide library wherein at said residue position a subgroup of the group of amino acids which has been identified as contributing to the optimal binding activity at said residue position is used, wherein the amino acids of the subgroup are added at said residue position at different molar concentrations;

(e) screening the third peptide library for binding to the ligand of interest and comparing the binding activity thereof to the baseline activity of the first or second library, thereby identifying the amino acids in the subgroup which contribute to optimal binding to the ligand of interest;

(f) synthesizing one or more additional soluble peptide libraries wherein at said residue position a single amino acid is substituted for each of the amino acids in the subgroup which contributed to optimal ligand binding at said residue position;

(g) screening the additional libraries for binding to the ligand

of interest and comparing the binding activity thereof to the activity of the first, second or third library and thereby determining the amino acid at said residue position which contributes to optimal binding to the ligand of interest; and

repeating steps (b)-(g) to identify the amino acids which contribute to optimal binding to the ligand of interest at other residue positions of the peptide and thereby identifying a peptide which binds to a ligand on interest.

Lerner 5,601,992

1. A method for detecting the interaction between a peptide and a target, comprising:

(I) placing the target in a container;

(ii) overlaying the target with a substrate that is free of the target, the substrate constructed and arranged to permit only substantially localized diffusion of the peptides therein;

(iii) applying a plurality of beads to the surface of the substrate in a manner such that the beads are separated from the target by the substrate and such that the beads are substantially spaced-apart from one another and are immobilized on the substrate, each bead having associated therewith multiple copies of only a single peptide, wherein at least a substantial portion of the multiple copies is associated with each bead via non-covalent bonds;

(iv) allowing the substantial portion of the multiple copies to diffuse through the substrate and interact with the target, thereby resulting in a localized signal at the target;

(v) detecting the localized signal; and

(vi) associating the localized signal with one or a limited number of the plurality of beads applied to the substrate.

Lebl 5,688,696

1. A method of making a combinatorial library of test compounds which comprises at least two successive iterations of the steps of:

a) selecting a set consisting of two or more types of subunits;

b) dividing a continuous solid phase support having a linker,

said linker having reactive sites and having one or more protecting groups, into a number of physically separate pieces of support equal to an integral multiple of the number of selected types of subunits;

c) attaching one type of subunit to substantially all the reactive sites attached to a corresponding piece of solid phase support; and thereafter

d) removing a protecting group from the linker so that reactive sites are provided,

thereby providing at least two subunits attached to each piece of solid phase support, and wherein each piece of solid phase support has a single species of test compound of selected subunits attached to it and each species of test compound is represented on a predetermined number of pieces of solid phase support.

Kahne 5,635,612

1. A method of forming multiple regioselective glycosidic linkages in a single step comprising:

(a) providing a bifunctional first glycoside (FG) in an organic solvent, said FG having (i) an anomeric sulfoxide substituent, and (ii) glycosyl accepting and glycosyl donating characteristics;

(b) treating said FG with an effective amount of an activating agent (AG), which AG renders the anomeric carbon to which said sulfoxide group is attached susceptible to nucleophilic attack; and

(c) stirring the resulting mixture to form regioselectively in a single step two or more glycosidic bonds linking three or more glycosides.

6355490

1. A process of coding individual members of a combinatorial chemical library synthesized on a plurality of solid supports comprising covalently attaching to each of the solid supports a coding identifier detectable by infrared or Raman spectroscopy, wherein said coding identifier attached to the solid support is detectable by infrared or Raman spectroscopy and said coding identifier is a nitrile.

Dower 5,639,603

1. A method of screening a tagged library of diverse compounds, wherein said library comprises a plurality of different members, each member comprising:

a solid support;

multiple copies of a compound bound to each of said supports, wherein the compound bound to one of said supports is different from the compound bound to selected other solid supports, and wherein said compound comprises a peptide; and

one or more identifier tags bound to each of said solid supports, wherein said tag identifies the compound bound to said solid support or identifies a reaction said solid support has experienced, and wherein said tag is an oligonucleotide or a fluorescent tag, said method comprising the steps of:

a) cleaving at least a portion of said compounds from said solid supports to yield a collection of untagged soluble compounds wherein said tags remain bound to said solid supports;

b) incubating said collection of untagged soluble compound s with a receptor under conditions conducive to binding of a ligand to said receptor; and

c. c) determining whether any compounds of said collection have bound to the receptor.

Schumacher 5,780,221

11. A method of identifying an L amino acid peptide which binds a D amino acid peptide of interest, comprising the steps of:

a) providing a phage display library which comprises L amino acid peptides displayed on phage surfaces;

b) contacting the phage display library of a) with the D amino acid peptide of interest, under conditions appropriate for binding of L amino acid peptides displayed on phage surfaces with the D amino acid peptide of interest; and

c) identifying phages on the surfaces of which the D amino acid peptide of interest is bound to an L amino acid peptide displayed on the surface, thereby producing a D amino acid peptide-displayed L amino acid peptide complex

wherein the displayed L amino acid peptide is an L amino acid peptide which binds the D amino acid of interest.

Schatz 5,733,731

1. A method of isolating a DNA binding protein comprising:

(a) providing a recombinant DNA vector comprising a coding sequence for a peptide having a specific affinity for a receptor;

(b) inserting a library of oligonucleotides encoding different potential DNA binding proteins into multiple copies of the recombinant DNA vector in-frame with the peptide coding sequence to form a library of different vectors encoding different fusion proteins, the fusion proteins differing in the potential DNA binding proteins;

(c) transforming host cells with the library of different vectors to form transformed host cells;

(d) culturing the transformed host cells under conditions suitable for expression of the fusion proteins, whereby, if a fusion protein comprises a potential DNA binding protein with affinity for a vector encoding the fusion protein, the fusion protein binds to the vector to form a complex;

(e) lysing the transformed host cells under conditions such that complexes formed in (d) remain associated;

(f) contacting the complexes with a receptor under conditions conducive to specific binding of the peptide to the receptor;

(g) isolating complexes bound to the receptor, the complexes containing vectors encoding DNA binding proteins.

Rutter 5,734,018

1. A method of obtaining molecules comprising a sequence of residues and having a desired target property, said method comprising the steps of:

providing a mixture of molecules comprising a sequence of residues wherein the mixture comprises 8,000 or more molecules having distinct, unique and different sequences of residues which are selected from the group consisting of naturally occurring amino acids and non-coded amino acids, wherein the sequence of residues of each molecule in the mixture is predetermined and

each molecule is present in an amount such that each molecule is analyzable; and

selecting from among the mixture of candidate molecules those molecules having a desired target property by exposing the mixture of candidate molecules to a substance to which the molecules having a desired target property will preferentially bind.

9. The method as claimed in claim 8, wherein the mixture of candidate molecules contains 64,000,000 or more different molecules.

kauffman 5,723,323

1. A method of identifying a peptide, polypeptide or protein having a binding property to a ligand, comprising:

(a) providing a ligand for detecting said binding property;

(b) synthesizing a diverse population of stochastically generated polynucleotide sequences;

(c) inserting said diverse population of stochastically generated polynucleotide sequences into a population of expression vectors to form a diverse population of expression vectors containing stochastically generated polynucleotide sequences;

(d) expressing in host cells said diverse population of expression vectors containing stochastically generated polynucleotide sequences to produce a diverse population of peptides, polypeptides or proteins; and

(e) screening said diverse population of peptides, polypeptides or proteins with said ligand under conditions which allow binding and detection of one or more peptides, polypeptides or proteins having said predetermined property.

2. The method of claim 1, wherein said stochastically generated polynucleotide sequences further comprises all twenty amino acid residues encoded at each codon position.

3. The method of claim 1, wherein said diverse population of stochastically generated polynucleotide sequences, encode at least 10,000 different peptides, polypeptides or proteins.

Kalenbach, 5,756,323

5. A method for generating structural diversity in a peptide sequence by randomly deleting or inserting nucleotides in a nucleotide sequence which codes for the peptide sequence, said method comprising the transfection of a mammalian cell preparation with one or more vectors expressing the products of the Rag-1 gene, Rag-2 gene and the terminal deoxynucleotidyl transferase (TdT) gene and by an identical or different vector including said nucleotide sequence bordered by one or more recombination signal sequences (RSS) to produce a recombined vector or vectors that express said nucleotide sequence to produce a mutated peptide.

5,750,373

1. A method for selecting novel binding polypeptides comprising:

(a) constructing a replicable expression vector comprising

a transcription regulatory element operably linked to a gene fusion encoding a fusion protein wherein the gene fusion comprises

a first gene encoding a polypeptide, and

a second gene encoding at least a portion of a wild-type M13 phage gene III coat protein;

(b) mutating the vector at one or more selected positions within the first gene thereby forming a family of related plasmids;

(c) transforming suitable E. coli host cells with the plasmids;

(d) infecting the transformed host cells with a M13K07 helper phage having a gene encoding the wild-type M13 phage gene III coat protein;

(e) culturing the transformed infected E. coli host cells under conditions suitable for forming recombinant phagemid particles containing at least a portion of the plasmid and capable of infecting the E. coli host, the phagemid particles displaying a mixture of the wild-type M13 phage gene III coat protein and the fusion protein;

(f) contacting the phagemid particles with a target molecule so that at least a portion of the phagemid particles bind to the target molecule; and

(g) separating the phagemid particles that bind from those that

do not.

Esshhar 5,766,861

1. A method for screening or detection of a catalytic polypeptide or protein suitable for the conversion of a substrate S to a product P, said method comprising:

i. immobilizing said substrate S to a support;

ii. contacting a preparation comprising a potential catalytic polypeptide or protein with the immobilized substrate S, thus converting the substrate S completely or partially to a product P, which remains immobilized to the support;

iii. detecting the immobilized product P molecules with antibodies specific to said product P; and

iv. detecting the product P-bound antibody molecules, the formation of product P at a rate and amount significantly higher than in a control reaction indicating the presence of said catalytic polypeptide or protein in the screened preparation.

Dower 5,723,286

1. A method for identifying a peptide which binds to a preselected receptor molecule, comprising:

transforming host cells by electroporation with at least 10.sup.8 different bacteriophage expression vectors, wherein each of said different vectors encodes a fusion protein comprising a peptide fused to a coat protein of a filamentous bacteriophage so that the N-terminal amino acid of said fusion protein is the N-terminal amino acid of said peptide, and wherein said different vectors are constructed by ligating each oligonucleotide of a mixture of at least 10.sup.8 different oligonucleotides to a bacteriophage cloning vector that encodes the coat protein so as to form a coding sequence for said fusion protein, wherein each of said different oligonucleotides comprises a series of codons encoding a random collection of amino acids and encodes a different peptide;

cultivating the transformed cell under conditions suitable for expression and assembly of bacteriophage particles thereby displaying said peptides on the surface of said particles; and

selecting bacteriophage particles displaying the peptide by

combining said particles with the preselected receptor molecule and separating particles bound to said receptor molecule from unbound particles.

Schultz 6346423

1. A method for synthesizing a plurality of polymers on a substrate surface, said method comprising:

(a) producing a solvent layer on said substrate surface, where said substrate surface has a plurality of individually activatable resistors associated with it;

(b) performing at least two iterations of the following steps:

(1) selectively protecting at least one site on said substrate surface with a protective bubble by selective activation of said resistors;

(2) contacting said selectively protected substrate surface with a reactive agent under conditions sufficient for said reactive agent to react with unprotected susceptible moieties present on said substrate surface; and

(3) removing unreacted reactive agent from said substrate surface;

whereby a plurality of polymers are produced on said substrate surface.

Kay, 5,948,635

1. A method for identifying a protein, polypeptide and/or peptide which binds to a ligand of choice, comprising: screening a library of recombinant vectors which express a plurality of heterofunctional fusion proteins comprising

(a) a binding domain encoded by an oligonucleotide comprising unpredictable nucleotides in which the unpredictable nucleotides are arranged in one or more contiguous sequences, wherein the total number of unpredictable nucleotides is greater than or equal to about 60 and less than or equal to about 600; and wherein the coding strand of the unpredictable nucleotides comprises the formula (NNB).sub.n+m where

N is A, C, G or T;

B is G, T or C; and

n and m are integers, such that

2.ltoreq. n+m.ltoreq.200; and

(b) an effector domain encoded by an oligonucleotide sequence encoding a protein or peptide that enhances expression or detection of the binding domain,

by contacting the plurality of heterofunctional fusion proteins with said ligand of choice under conditions conducive to ligand binding and isolating the fusion proteins which bind said ligand.

Ruoslahti 6,068,829

1. A method of recovering a plurality of peptide or peptidomimetic molecules that home to a selected organ or tissue, comprising the steps of:

a. administering to a subject a library of diverse peptide or peptidomimetic molecules, wherein each of said diverse molecules is linked to a tag that facilitates recovery of said peptide or peptidomimetic molecules;

b. collecting a sample of the selected organ or tissue; and

c. recovering a plurality of peptide or peptidomimetic molecules that home to said selected organ or tissue by isolating molecules comprising said tag from said sample.

14. A method of identifying a peptide or peptidomimetic molecule that homes to a selected organ or tissue, comprising the steps of:

a. administering to a subject a library of diverse peptide or peptidomimetic molecules, wherein each of said diverse peptide or peptidomimetic molecules is linked to a unique oligonucleotide tag;

b. collecting a sample of the selected organ or tissue; and

c. identifying a unique oligonucleotide tag present in said sample, thereby identifying a peptide or peptidomimetic molecule that homes to said selected organ or tissue.

21. The method of claim 14, wherein said library comprises several billion or more diverse peptide or peptidomimetic molecules.

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Microbial interaction with animal cell surface carbohydrates

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Microbes have selected primarily carbohydrates for attachment to host animal cells. Recent studies have revealed essential characteristics in the recognition of receptor carbohydrates. Of importance is the property of recognizing also sequences placed inside an oligosaccharide chain, which differs from most animal antibodies. This is the basis for series of isoreceptors with the minimum receptor sequence in common but with separate neighbouring groups. There are families of microbial ligands that show different preferences for members within one series of isoreceptors, indicating only slight differences in the complementary binding sites of the proteins. Such differences may explain shifts in the selectivity of separate host tissues for infection. A second characteristic is the low affinity interaction often found where simple receptor-containing saccharides are unable to inhibit attachment. Technical possibilities are rapidly developing for the design of synthetic receptor analogues to be used in the therapy of clinical infections. This is urgently needed in cases where no rational therapy exists today.

Key words: Microbe; bacteria; virus; toxin; carbohydrate; glycosphingolipid; receptor; adhesion; binding epitope; epitope dissection; adhesin; therapy.

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The abundance of carbohydrates in various forms at the animal cell surface is one reason why microbes to a large extent have selected carbohydrate receptors for colonization and infection. Glycolipids play a special role since they are usually strictly membrane-bound and do not appear in secretions as potential inhibitors of membrane attachment, as do glycoproteins. They are also easier to handle technically than protein-bound oligosaccharides as there is only one oligosaccharide for each molecule. This allows a rational binding assay based on overlay of a virus (8) or bacterial (9) suspension on thin-layer chromatograms with separated molecular species of glycolipids. Many microbes have, in fact, selected relatively glycolipid-specific receptors (lactose, and glycolipid members of the ganglio and globo series). The development during the last few years of assay, separation and structural techniques for glycoconjugates, in combination with knowledge from microbial molecular biology, is at present contributing information on both prokaryotic and eukaryotic protein-carbohydrate interactions that hold promise also for drug design (14).

The purpose of the present communication is to summarize and discuss important characteristics of glycolipid receptors for microbes with emphasis on recent results from our own laboratory and extrapolation to biomedical applications. The recognition by microbes of internal sequences of the saccharide chain and the potential membrane proximity of the binding are two properties of more conceptual interest.

THE GENERAL PROPERTY OF MICROBES TO RECOGNIZE NON-TERMINAL RECEPTOR SEQUENCES IS THE BASIS FOR FAMILIES OF RECEPTOR-BINDING VARIANTS

In contrast to animal antibodies that recognize carbohydrates, almost all of which bind to terminal sequences (31), microbial proteins have been convincingly shown to bind also to internal epitopes of receptor saccharides (13, 18). This is true for viruses, bacteria and bacterial toxins and this property will appear as one characteristic in the following presentation.

When using the rational overlay techniques (8, 9, 15) to detect glycolipid-based receptors, a particular ligand may bind to a large number of receptor-active bands on the thin-layer plate (3). Of these, only a few may have the active sequence in the terminal position (3). Also, some glycolipids which carry the active sequence in internal position may be receptor-inactive, based on non-accessibility of the binding epitope due to steric hindrances from neighbouring groups or for other reasons. We have defined the name *isoreceptors* for oligosaccharide sequences, regardless of whether or not they are active for a particular ligand, which have the minimum receptor sequence in common but placed in different environments (flanked by separate neighbouring groups). A large number of bacteria, bacterial toxins and viruses have already been analysed using this overlay technique. An interesting grouping of receptor specificities into families has been revealed (13, 18), where each family consists of ligands that require the same minimum receptor sequence but where individual members may show different preferences for individual isoreceptors. Examples of such families are given in Table 1.

Within each family a minimum sequence is required but there is separate dependence on flanking sequences or ceramide structure. The conclusion has been that the binding epitope on the isoreceptors varies slightly between variant binders and that the complementary binding sites of the receptor-binding proteins are structurally and evolutionary related.

The biological meaning of the internal binding and these variants has been briefly discussed (13), and there is increasing experimental evidence to support the given proposal. The mutational effort to shift specificity between two isoreceptors was considered limited (a few amino acids in the binding site) compared to a shift between two families of isoreceptors. The internal binding is essential as the basis for isoreceptors

TABLE 1. *Families of receptor-binding variants of microbial ligands. Within one family, variants show different binding preferences to isoreceptors, which have a minimum receptor sequence in common. The information has been taken mainly from Refs. 2, 13, 18, 19, 26, 27, 28, 29.*

Lactosylceramide binders R1-*Galβ4Glc*-R2	A large number of bacteria have been shown to bind to separate patterns of isoreceptors with lactose as minimum requirement. The bacteria are both normal flora, mainly large intestine, and important pathogens such as *Bordetella pertussis, Vibrio cholerae, Shigella dysenteriae, Neisseria gonorrhoeae.* Among the isoreceptors are lactosylceramide species with separate ceramide structures and these are illustrated in the present paper (Table 3).
Galabiose binders R1-*Galα4Gal*-R2	Several variant adhesins of different *Escherichia coli* strains causing urinary tract infection in human and dog. Shiga-like toxins.
Sialic acid binders R1-*NeuAc*-R2	Although not yet established, this is probably a very large family, including several viruses, bacteria and bacterial toxins. The illustration in the present paper shows cholera and tetanus toxins, and their different but related binding epitopes on isoreceptors (Fig. 8).
Monoglycosylceramide binders R1-*HexβCer*	A group of viruses have been shown to bind with this specificity. These belong to *Adenoviridae, Herepetoviridae, Orthomyxoviridae, Paramyxoviridae, Myxoviridae, Rhabdoviridae, Reoviridae* and *Retroviridae*, including for example influenza and AIDS virus. The two binding variants Sendai virus and HIV-1 will be illustrated in the present paper (Table 6).

TABLE 2. *Relation between different binding to isoreceptors on cell membranes and tissue tropism in urinary tract infections in human and dog. Data are taken from Refs. 3, 18, 20, 21 and 29. For isoreceptor sequences, see Figs. 1 and 2.*

	Globoside	Forssman	Globo A
Binding by			
Class II adhesin	+	–	–
Class III adhesin	–	+	+
Isoreceptor presence in urinary tract of			
Dog	–	+	?
Blood group A-positive humans	+	–	+
Blood group A-negative humans	+	–	–
Binding to epithelial cells of human urinary tract when *only* Class III adhesin is expressed by *E. coli* isolate			
Blood group A1 secretors	–	–	+
Blood group A1 non-secretors, blood group A2 or blood group A-negative	–	–	–
	Class II Adhesin	Class III Adhesin	
Frequency of expression of adhesin in *E. coli* isolates of			
Human	+++	+	
Dog	+	+++	

with only subtle differences at the binding epitope; a terminal binding, with a neighbour only on one side, would most probably require a greater effort for a shift to occur. Also, the main difference between isoreceptors lies within the neighbour most distal from ceramide. To have biological significance, it must be possible to correlate this main characteristic of microbes with virulence properties. In the following, two examples will be shown where a shift in tissue tropism may be explained on the basis of variant binders. The first is *E. coli* and urinary tract infection and a shift between human and dog, and the second is lactosylceramide binders and epithelial and non-epithelial localization. The first will now be discussed, and the second later in this presentation.

Urinary tract infection and shift in tropism between human and dog

As summarized in Table 2, there is an interesting finding where a change in colonization between human and dog may be explained by a receptor shift beween two isoreceptors within the galabiose family (Table 1). In the first detailed characterization of a carbohydrate-binding specificity for a bacterium published in 1985 (3), a clinical isolate of *E. coli* from human urinary tract infection was shown to recognize on thin-layer plates all known isoreceptors within the galabiose glycolipid family (globo series of glycolipids). It has later been found through extensive work on cloning variant adhesins and testing these for receptor specificity combined with frequency of expression in urinary tract infection (29), that a shift in binding between globoside (Class II adhesin) and Forssman glycolipid (Class III adhesin) may explain colonization in the urinary tract of human and dog, respectively. Furthermore, it has been shown that human isolates of *E. coli* that express only Class III adhesin appear in individuals of blood group A only (20). This may be explained (18, 29) by a dominance of globoside (binding by Class II adhesin) in the human and a dominance of Forssman glycolipid (binding by Class III adhesin) in the dog. The difference in binding epitope between Class II and Class III adhesins is probably that Class II requires Galβ in GalNAcβ3Galα4Galβ, while Class III has an epitope shifted to the left and not requiring Galβ. This is illustrated in Fig. 1, where calculated conformations of the molecules as they probably appear in the surface membrane are projected for globoside and Forssman glycolipid (left and right, respectively) with indicated arbitrary binding epitopes. The reason why Class II does not bind to Forssman glycolipid is that the Galβ

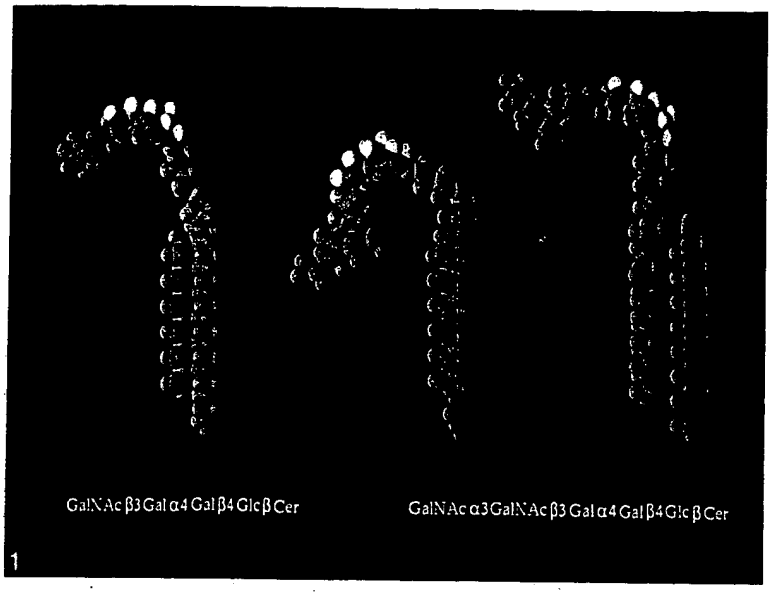

Fig. 1. Molecular models of globoside (left), and Forssman glycolipid (in two separate conformations). The non-polar ring hydrogens of Galα4Gal are shown in white and the rest of the molecules in grey. The purpose is to illustrate the probable basis for the tropism of urinary tract infection in human and dog, respectively (compare with binding data of Table 2). The Class II adhesin of *E. coli*, which is most frequent in human infections, binds selectively to globoside on cell membranes. The Class III adhesin, however, which is most frequent in dog infections, binds selectively to Forssman glycolipid. Globoside is typical of human cells and Forssman glycolipid of dog cells. Interestingly, this discrimination is not present when the two glycolipids are exposed on assay surfaces like thin-layer plates or microtitre wells. The explanation is that the slightly different binding epitopes for the two adhesins are present on both glycolipids but selectively hidden when placed on the membrane, though not on the assay surface. The models show that the Galα4Gal, which is required for Class II adhesin, is easily accessible in globoside (left) but not Forssman glycolipid (far right), when they are placed in the membrane. However, on assay surfaces, without restrictions from placement in the membrane, the conformation in the middle (one of several) is also possible, where the disaccharide is better presented. This demonstrates that membrane proximity of the binding epitope may influence the specificity. For modelling, see data in Table 4.

Fig. 2. Molecular model of blood group A globoside which is selectively recognized on cell membranes by Class III adhesin of *E. coli* in urinary tract infection (compare Table 2). It may be compared with the far right conformation of the Forssman glycolipid of Fig. 1. Although the binding epitope for Class II adhesin exists in this glycolipid, it is selectively shielded by the bending of the saccharide in the membrane location, compared with the non-membrane location (compare middle conformation in Fig. 1).

residue is forced down at the side in the Forssman glycolipid (and in the globo A glycolipid, which is visualized in Fig. 2) and is thus not accessible for binding. Class III, on the other hand, not requiring this Galβ (and not able to bind to sequences with terminal Galα4Gal, in contrast to Class II, see References 18 and 29) binds perfectly to this conformation. Why Class III practically does not bind to globoside in the membrane (but binds well on technical assay surfaces, see below) may be that the Class III epitope (as it exists on Forssman or globo A) is not properly presented.

The conclusion from this discussion is that the tropism of infection may be shifted by a minimum change in the binding site of the adhesin (not yet localized in the cloned sequences), and that this determines a change in selectivity for isoreceptors on the target cell membrane. A different appearance of isoreceptor patterns between cells, which is often found, is the basis of the targeting to specific cells.

THE DEPENDENCE OF SPECIFICITY ON CERAMIDE STRUCTURE: MEMBRANE PROXIMITY AND CONFORMATION

Galabiose series of receptors

The proximity of a carbohydrate binding epitope to ceramide may have effects on the selectivity of microbial binding to membrane-located glycolipids due to the constraints produced by the bilayer surface. These constraints are not present when the glycolipid appears in a non-membrane environment, as on technical assay surfaces. A convincing example of this exists within the galabiose family of binders (see the discussion above). Class II adhesin binds about equally well to globoside and to Forssman glycolipid or globo A glycolipid when they are presented on thin-layer chromatograms or in microtitre wells. However, on intact cells only globoside is recognized (29). A reason for this is illustrated in Fig. 1, where the Forssman glycolipid is presented in two low-energy conformers, one of which may exist on non-membrane surfaces but is unlikely to exist in membranes (middle), and one of which may exist in the membrane (right). If Class II adhesin is dependent on accessibility of Galβ in galabiose for binding, this part will then be exposed in the centre conformer but not in the right one due to the bending of the saccharide forced by the membrane location. In the right conformer this sequence is usually presented to the side in the membrane. Thus the membrane location produces a higher selectivity in binding to glycolipids than when these appear on technical surfaces. Logically, the presentation that the bacteria select for an optimal colonization should be the membrane-dependent presentation on target cells. The membrane proximity may also be important for penetration (see discussion below on second-step receptors).

Lactosylceramides

An example of dependence of epitope conformation on ceramide structure regardless of membrane location, is the family of lactose binders (Table 1). As shown in Table 3, there are two groups of members depending on which type of lactosylceramide they recognize. Interestingly, this may be related to

TABLE 3. *Members of the lactosylceramide-binding family (Table 1) that select lactosylceramides, Galβ4GlcβCer, based on differences in ceramide structure. Data taken from Refs. 11, 13, 18, 27 and 28.*

	LacCer with sphingosine and non-hydroxy acid	LacCer with sphingosine and hydroxy acid or phytosphingosine and hydroxy acid
Propionibacterium freudenreichii and several yeasts and fungi	++	–
Normal flora of large intestine and several pathogens, see Table 1	–	++

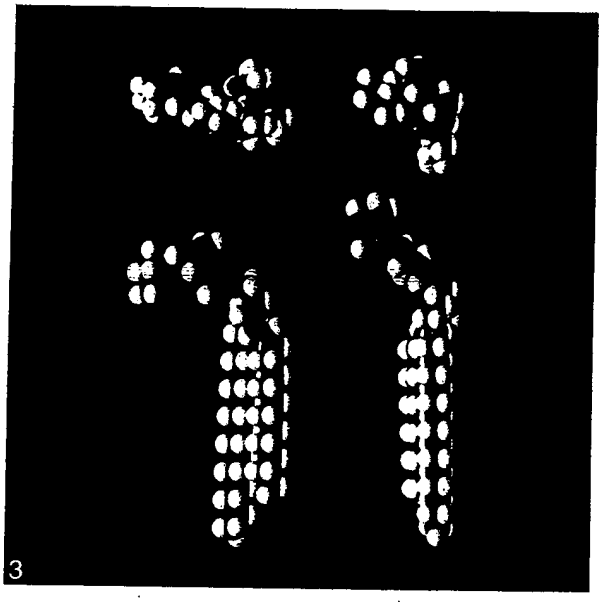

Fig. 3. Molecular models of low-energy conformers of lactosylceramide having separate ceramide structures and being selectively recognized by microbes (compare with binding data of Table 3). The left species is LacCer with C16 sphingosine and 2-D-hydroxy 18:0 fatty acid, and the right species LacCer with C16 sphingosine and non-hydroxy 18:0 fatty acid. O-6 of Gal and Glc have been labelled with a purple colour, and as can be seen O-6 of Gal is not accessible for binding in the left conformer in contrast to the right conformer. (This is best seen in the two top projections which show the surfaces of a membrane location which are exposed to the outside). The polar side of Gal is exposed in the left conformer and the non-polar side in the right conformer. It is likely that binding to these two sides explains the selectivity with no cross-binding by the two groups of microbes (Table 3). For modelling conditions, see Table 4.

TABLE 4. *Minimum energy conformers of lactosylceramide and sulfated galactosylceramide with 2-D-hydroxy fatty acid or non-hydroxy fatty acid*[a]

Conformer	Relative energy (kcal/mol)	HexβCer dihedral angles[b] Φ	Ψ	θ	HexβCer hydrogen bonds[c]
LacCer with 2-D-OH acid					
1*	0	13	-90	-59	O-2 ··· HN, OH-2 ··· O'-2
2	5.0	51	-179	67	OH-2 ··· O-2, O-1 ··· HO-2
3[d]	5.1	48	-138	63	OH-2 ··· O-2, O-1 ··· HO-2
4	7.7	47	-74	-172	OH-2 ··· O-1
5[e]	9.0	41	-93	165	OH-2 ··· O-1
6	10.4	51	180	177	–
LacCer with non-OH acid					
1	0	47	177	66	OH-2 ··· O-2, O-1 ··· HO-2
2	1.8	15	-90	-61	O-2 ··· HN
3*	2.2	50	-74	-176	OH-2 ··· O-1
4[f]	2.7	47	-122	65	OH-2 ··· O-2, O-1 ··· HO-2
5	3.7	40	-97	167	OH-2 ··· O-1
6	4.6	48	177	175	–
SO_3-3GalCer with 2-D-OH acid					
1*	0	12	-90	-59	O-2 ··· HN, OH-2 ··· O'-2
2	2.3	13	-91	-59	O-2 ··· HN, OH-2 ··· OS
3	2.7	56	-178	68	O-1 ··· HO-2, OH-2 ··· OS
4	4.0	39	-158	67	OH-2 ··· O-2, O-1 ··· HO-2
5	5.5	53	176	180	OH-2 ··· OS
6	5.7	54	-177	-63	OH-2 ··· OS
SO_3-3GalCer with non-OH acid					
1	0	14	-91	-60	O-2 ··· HN, OH-2 ··· OS
2	0.7	56	179	69	O-1 ··· HO-2, OH-2 ··· OS
3	2.8	50	173	-55	OH-2 ··· OS
4	3.1	43	-156	66	OH-2 ··· O-2, O-1 ··· HO-2
5	4.3	52	179	178	OH-2 ··· OS
6*	4.7	46	-75	-169	OH-2 ··· O'-1

[a] The relative energy of the minimum energy conformers of GalβCer is the same as listed above for LacCer. Minimum energy conformers of the various molecules shown in Figs. 1-5 and 8 were calculated using the DREIDING force field (23) within the Biograf molecular modelling software package from Molecular Simulations Inc. placed on a Silicon Graphics 4D/25TG workstation.

[b] The hexose-ceramide dihedral angles are defined as follows: Φ = H-1 - C-1 - O-1 - C-1, Ψ = C-1 - O-1 - C-1 - C-2, and Θ = O-1 - C-1 - C-2 - C-3.

[c] Hydrogen bonds are listed with the hexose donor (acceptor) first. In several cases, however, the OH-2 of Gal preferably forms a hydrogen bond with the sulfate group in the sulfatide, which for the sake of completeness is also given. Hydrogen bond energies are in the order of -5 to -8 kcal/mol. Substituents on the fatty acid have been primed.

[d] Strained conformer of no. 2.

[e] Strained conformer of no. 4.

[f] Strained conformer of no. 1.

* Denotes conformers shown in Figs. 3-5.

the tropism of infection, since lactosylceramide with less hydroxylated ceramide is present in non-epithelial cells, while that with the higher level of hydroxylation is present in epithelial cells lining the mucous surfaces. The level of hydroxylation has been proposed to affect membrane stability (12). There is no evidence that the ceramide is part of the binding epitopes. It is more likely that the lactose moiety shows different sides for binding, as depicted by molecular modelling in Fig. 3 (see also Table 4 for energy levels). Consider for example OH-6 of Gal, which is available for binding in the low-energy conformer of the less hydroxylated species (right), whereas it is pointing downwards in relation to the membrane surface in the conformer of the other species (left). Therefore, when the two conformers are compared, the disaccharide is presented differently to a potential binder, with the non-polar side of Gal available in the right conformer but the polar side of Gal in the left one. It is hence likely that the two groups of ligands recognize separate epitopes on lactose with no cross-binding. Furthermore, the members which selectively bind the hydroxylated species can also bind to several extended sequences (e.g. Galα3, GalNAcβ4, Galβ3GalNAcβ4), which members binding the non-hydroxylated forms are unable to do (13, 27). Within the former group there are variants which show different preferences in binding to extended sequences of lactosylceramide (13, 18).

Hexosylceramides

Using the thin-layer chromatographic overlay method (8) we have demonstrated that several non-related viruses carry a common receptor specificity based on glycolipids (16, 18). Recently, a related specificity was also found for the AIDS virus, HIV-1 (2, 10). The actual viruses are gathered in Table 5.

As shown in more detail for Sendai virus (16, 18), HexβCer may be extended by up to four sugars with retained binding. However, only some extensions are positive, probably due to steric hindrance or other causes of non-accessibility of the binding epitope. Thus, also viruses carry the property of binding internally in a saccharide chain. Except for HIV-1, both GlcβCer and GalβCer are receptor-active. However, there is a dependency on ceramide structure analogous to the case for lactosylceramide binders (see discussion above with respect to Tables 3 and 4, and Fig. 3). Except for HIV-1, the viruses of Table 5 bind exclusively to HexβCer species with sphingosine combined with hydroxy fatty acid or phytosphingosine combined with hydroxy fatty acid. HIV-1, on the other hand, differs by also binding to species with non-hydroxy fatty acid, but only to GalβCer and not to GlcβCer. HIV-1, in addition, binds to sulfatide, which Sendai virus does not. These data are summarized in Table 6. (One reservation should be expressed

TABLE 5. *Viruses that have been shown to carry a common glycolipid-binding specificity with a minimum sequence of one sugar. Data were taken from Refs. 2, 10, 16 and 18.*

Member	Classical receptor	Additional specificity
Adenovirus 2, 7	Peptide	HexβCer
B95-8 EB virus	B-cell peptide	HexβCer
Influenza virus	Sialic acid	HexβCer
Mumps virus	Sialic acid	HexβCer
Sendai virus, two variants	Sialic acid	HexβCer
Rabies virus ERA	?	HexβCer
Rotavirus K8	Carbohydrate	HexβCer
Reovirus 1, 2, 3	Carbohydrate?	HexβCer
HIV-1	CD4-peptide	HexβCer

TABLE 6. *Comparison of Sendai virus (16, 18) and HIV-1 (2, 10) with respect to binding to one-sugar glycolipids using the thin-layer chromatogram overlay method*

	Sendai Virus	HIV-1
GlcβCer with sphingosine and hydroxy acid or phytosphingosine and hydroxy acid (Left conformer of Fig. 5)	+	–
GalβCer with sphingosine and non-hydroxy acid (Right conformer in Fig. 4)	–	+
GalβCer with sphingosine and hydroxy acid or phytosphingosine and hydroxy acid (Left conformer in Fig. 4)	+	?
Sulfatide (SO_3-3GalβCer, centre and right conformers in Fig. 5)	–	+
Extensions of GlcβCer, eg. Galβ4GlcβCer, Galβ3(Fucα4)-GlcNAcβ3Galβ4GlcβCer	+	–

as to the details respecting these differences: HIV-1 on the one hand, and the other viruses on the other, have been characterized by separate laboratories. Until they have been compared under exactly the same conditions, no definite conclusions can be drawn.)

In the following we will present a preliminary interpretation of probable differences in binding epitopes between Sendai virus and HIV-1, based on calculated low-energy conformers of various isoreceptors (Figs. 4 and 5). The two models, hydroxylated and non-hydroxylated, of GalβCer of Fig. 4 are analogous to those of lactosylceramide in Fig. 3. One conclusion was that part of the ceramide is included in the recognized epitope of the hydroxylated form and that the non-polar side of Hex is involved in the binding of viruses which are not HIV-1 (16, 18). This is visualized in the left conformer in Fig. 4, where both part of the ceramide and the non-polar ring hydrogens of Gal are accessible on the top projection. A reason why also GlcβCer is active may be seen from this conformer, where a change in configuration of O-4 (yellow) to Glc

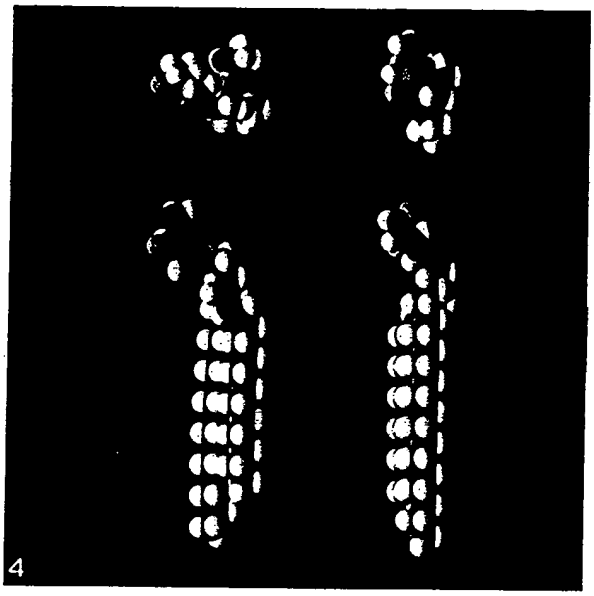

Fig. 4. Molecular models of low-energy conformers of GalβCer having separate ceramide structures, the left being recognized by several viruses which are not HIV-1, and the right being recognized by HIV-1 (compare binding data of Table 6). The two species correspond to the ceramide structures of LacCers of Fig. 3. Viruses that bind the left species also recognize GlcβCer, which is not the case for HIV-1. The O-4, which differs in configuration between Gal and Glc, is indicated in a separate yellow colour, as is O-3 (purple), which is the position for a sulfate group (compare full-colour models of GlcβCer and sulfatides of Fig. 5). One may envisage that O-4 has a more critical exposition in the right conformer and that change to Glc-O-4 may disturb a directed hydrogen bonding. Also, a sulfate group at O-3 is tolerated by HIV-1, which recognizes a polar side of Gal. In contrast, the other viruses probably interact mainly with a non-polar side of Gal or Glc (best seen in the top projection to the left) and do not tolerate a sulfate group in position 3. On the other hand, the configuration of O-4 is critical for HIV-1 but not for the other viruses. Therefore, the two groups of viruses are variants of HexβCer binders. For modelling conditions, see Table 4.

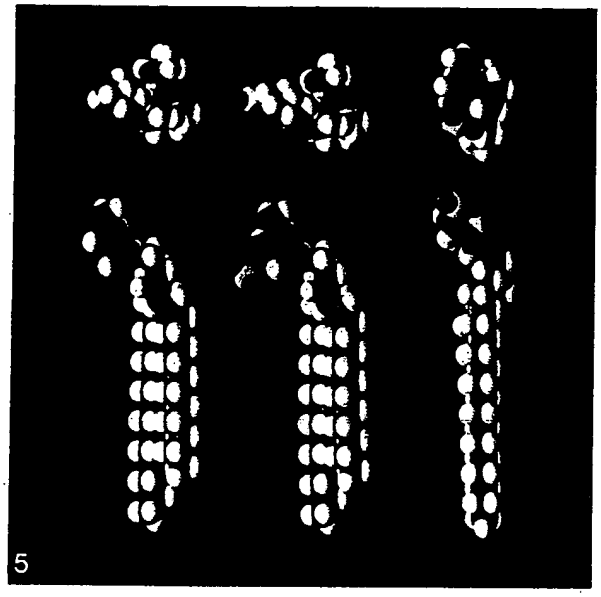

Fig. 5. Low-energy conformers of GlcβCer (same ceramide as GalβCer of Fig. 4, left), and two species of sulfatide, SO_3-3GalβCer, corresponding to the two conformers of GalβCer of Fig. 4. Sulfatide is recognized by HIV-1 but not by the other viruses of Table 5. On the other hand, GlcβCer is not recognized by HIV-1, but by the other viruses. One may envisage that the sulfate group interferes with a mainly non-polar interaction for non-HIV-1 viruses (left conformer of Fig. 4), while it is part of the more polar side being recognized by HIV-1 (compare right conformer of Fig. 4). O-4 on GlcβCer (left) does not interfere with binding by non-HIV-1 viruses and may be extended with further sugars without interfering with binding. In contrast, HIV-1, which may have a directed hydrogen bonding with OH-4 of Gal, does not accept an inverse configuration of this group. Compare legend of Fig. 4. For modelling conditions, see Table 4.

may not be critical for accessibility of the binding side (see also Fig 5, left). On the other hand, in the binding by HIV-1 to the right conformer in Fig. 4, the configuration of OH-4 may be critical, if it, as indicated, is exposed on the binding side and may be involved in hydrogen bonding. A less polar interaction in the case of Sendai virus may explain why sulfatide is inactive (position 3 for the sulfate group is indicated in purple in Fig. 4 and corresponding sulfatide models are shown in Fig. 5). In contrast, HIV-1 may carry a positively charged complementary site that tolerates the sulfate group in a neighbouring position to the postulated hydrogen bonding at OH-4. Thus, Sendai virus and HIV-1 are variant binders to isoreceptors of HexβCer. The biological meaning of this binding will be discussed below in relation to second-step receptors.

SECOND-STEP RECEPTORS BASED ON GLYCOLIPID SPECIFICITY AND MEMBRANE PROXIMITY: A HYPOTHESIS

Lactosylceramide-binding bacteria or HexβCer-binding viruses that colonize or infect separate cells or tissues are so diverse that they certainly cannot use a common binding specificity to effect these various tropisms. A characteristic feature seems to be the presence of at least two binding specificities for each particular microbe (see Table 5). A working model (13, 16) predicts that the infectious process involves a two-step mechanism (or in some bacterial cases a multistep mechanism). The first-step receptor mediates the targeting and tropism of the infection. The second-step receptor establishes a true cell-membrane attachment (lactose binders) or mediates the penetration into cells (some lactose-binding bacteria and HexβCer-binding viruses). For this to work, the second-step receptors used in common cannot be directly accessible from the outside of the cell, since this would destroy the selectivity. Therefore, the accessibility has to be induced after binding to the first-step receptor. Firstly, it has been established that one- and two-sugar glycolipids are not accessible for galactose oxidase labelling on normal cells (5, and discussion in 13). Secondly, a kinetic cell adhesion model (1) includes repulsion forces from an approaching particle (less likely for a molecule) that may induce a lateral diffusion and reorganization of surface components for a final lowest-energy binding. These data therefore support our model.

In the case of lactosylceramide-binding bacteria, it is of interest that lactose in bound form is known only in glycolipids, which are specifically membrane-associated and do not appear in secretions as do glycoproteins, which are potential inhibitors of membrane attachment. Bacteria may therefore secure a true cell adhesion by a second-step lactosylceramide receptor. Furthermore, several of these bacteria are invasive (13) and lactose binding next to ceramide may mediate an essential proximity to the membrane bilayer.

Viruses have to penetrate into the host cell to reproduce. Therefore the second-step binding to isoreceptors of HexβCer may give the proximity between virus and host cell membranes of less than 10 Å to induce membrane fusion, either at the plasma cell membrane or in the endosome. Although the biological relevance of the second-step mechanism has not yet been proven, the recent data from HIV-1 and blocking of both binding and infectivity, and localization of binding activity to gp120, are positive arguments (2, 10).

POTENTIAL APPLICATIONS

It is an old concept that in order to infect microbes have to specifically adhere to the target cell to avoid being eluted. In the case of viruses there is also a need to get into the cell. Applications where inhibition of attachment or adhesion can prevent or cure an infection in cases where no alternative is working in practice seem attractive. In the following some data relevant for anti-adhesion applications will be discussed.

The affinity of interactions

When trying to inhibit attachment there are two levels of inhibition, one where free oligosaccharides are able to inhibit and one where they are not able to inhibit . As an illustration, uropathogenic *E. coli* and Shiga toxin both recognize isoreceptors based on Galα4Gal (3, 19). How-

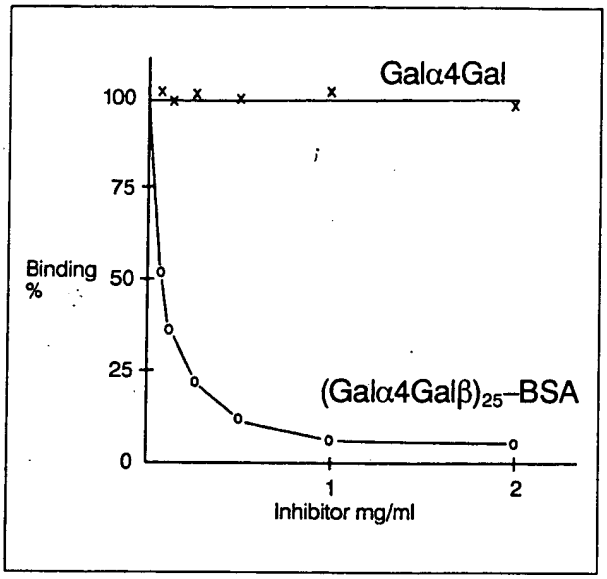

Fig. 6 Binding curves for inhibition of binding of Shiga toxin to Vero cells. Univalent Galα4Gal is unable to inhibit up to 2 mg/ml, while the same disaccharide linked multivalently to BSA (optimal with 25 residues per BSA molecule) is very efficient. Data taken from Ref. 19.

ever, while it is possible to inhibit *E. coli* attachment to target cells by univalent free oligosaccharides, this is not the case with Shiga toxin. As shown in Fig. 6, inhibition of binding of Shiga toxin (six or seven binding subunits) to Vero cells is not possible with relatively high concentrations of oligosaccharide. (At this level *E. coli* is inhibited *in vitro* in its adhesion to uroepithelial cells, although not completely.) However, when multivalently presented in conjugation with BSA, there is efficient inhibition. This is receptor-specific, since lactose linked in the same way is completely inactive. Binding to glycolipid receptors on thin-layer plates or in microtitre wells works well for both ligands due to a multivalent presentation. One conclusion from this is that the traditional inhibition assay or criterion of receptor specificity based on inhibition with free oligosaccharides is not applicable in some microbial protein-carbohydrate interactions. In fact, our impression at the present stage of knowledge is that a majority of systems have this low-affinity type of binding. Simple binding curves from binding of the ligand in microtitre wells coated with receptor-active glycolipid may define whether the ligand is of this type or not (15, 19, 28). The classical NeuAc receptor for influenza virus is of this type. Interestingly, several synthetic chemistry papers have appeared during the last year showing that NeuAc linked multivalently to different carriers are efficient inhibitors of virus binding (6, 7 , 22, 25).

Generally, the interaction between a virus, bacterium or parasite and the animal host cell is multivalent. For an inhibitor to be efficient in competing with this binding, there must be multivalency or, if univalent, the affinity of individual sites must probably be much higher than the natural affinity. For influenza virus, it is possible to inhibit with univalent receptor analogues where NeuAc is linked with a spacer to various aromatic structures (32). The best inhibitor was 64 times better than the methyl ketoside. Similarly, a conceptually important result is the thousand-fold improvement in efficiency of both inhibition of yeast cell agglutination and binding to intestinal epithelial cells of type 1 *E. coli* using aromatic residues linked to Manα (4). As a control, the corresponding Glcα derivative was completely inactive, showing that it is possible to improve an analogue this much with retained binding specificity. In this case it was even possible to elute already adhered bacteria.

In principle, a low-affinity interaction should mean an inefficient fit between protein and carbohydrate. Theoretically, it is possible to design an analogue with perfect fit to improve the interaction (Fig. 7). In cases with extremely high

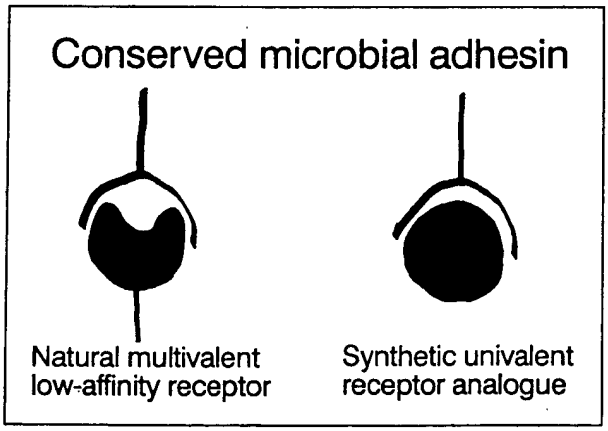

Fig. 7. Figure to visualize the assumed non-perfect fit of carbohydrate receptor and protein site (left) in case of low-affinity interactions (of the type shown in Fig. 6). On the right, an optimized synthetic analogue is visualized to have a better fit and thus be a much better binder than the natural receptor structure.

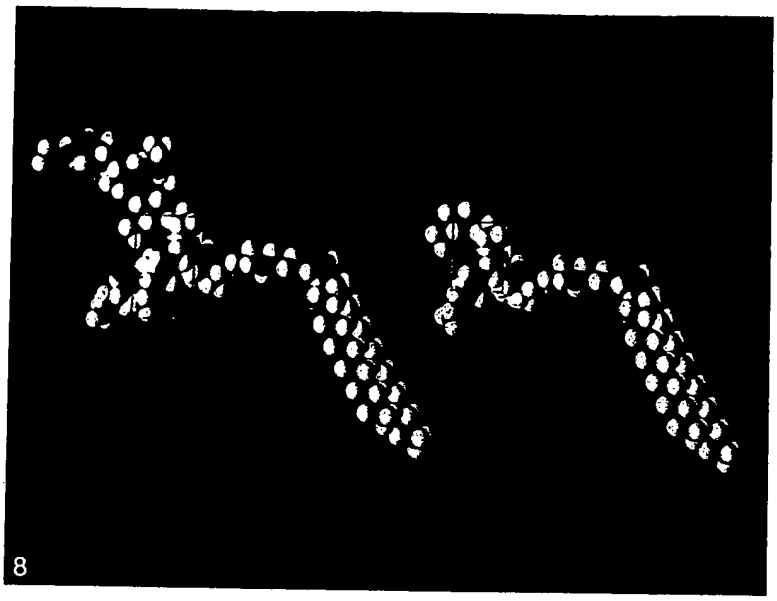

Fig. 8. Molecular models of the gangliosides GM1 (right) and GQ1b, which are optimal receptors for cholera toxin and tetanus toxin, respectively. The rectangles mark the probable binding epitopes, which differ but are highly overlapping based on the internal binding. Although the sequences differ, the epitopes are related and therefore the complementary binding sites of the two toxins should be structurally and evolutionary related. The sequences are for GM1: Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ-Cer, and for GQ1b: NeuAcα8NeuAcα3 Galβ3GalNAcβ4(NeuAcα8NeuAcα3) Galβ4GlcβCer. For modelling conditions, see Table 4.

affinities, as for periplasmic transport proteins of bacteria, crystal structures of complexes of protein and monosaccharide have shown that all possible interaction points in the binding site have been used, including directed hydrogen bonding and van der Waals interaction (33).

It is possible to use multivalent inhibitors without resorption on mucosal surfaces. However, in most cases a drug has to be resorbed through membranes and therefore has to be small enough and resist rapid elimination.

First proof of the concept of anti-adhesion for therapy

Recently, results were published that proved for the first time in a clinically relevant situation that anti-adhesion is possible (24). New-born calves were infected with a lethal dose of diarrhoea-inducing *E. coli* K99, and when symptoms of diarrhoea appeared they were given an oral dose of 1 - 3 g of oligosaccharides prepared from cow blood plasma, which was sufficient for cure. In parallel we showed (30) that the active receptor sequence for this bacterium is most probably limited to NeuGcα3Gal, and saccharides with this sequence were probably only part of the given dose. In relation to the fact that the small intestine, which is the target for these bacteria, has a total mucosal surface in the range of a soccer ground, these results are very promising. Theoretically, if the dose given contained 10% (at the most) of the receptor-active sequence, and, in analogy with the Manα derivative discussed above, an efficient synthetic analogue was used, the active dose could be reduced to 100 - 300 micrograms per animal.

Drug design

Rational drug design is considered based on a three-dimensional knowledge of the receptor-binding protein site, most optimally the receptor-protein complex. Only one such case has been resolved so far for attachment complexes, namely the crystal structure of influenza virus haemagglutinin in complex with sialic acid (34). Interestingly, the receptor carbohydrate did produce less sharp diffraction, indicating a looser position, in line with the low-affinity interaction.

Although the information on crystal structures of such complexes cannot yet be obtained to allow computer-based modelling of optimized synthetic low-molecular receptor analogues, it is possible to approximate binding epitopes on receptor saccharides without structural knowledge of the protein. This we have named epitope dissection (13); it is based on the property of microbes to bind internally in the chain to produce families of isoreceptors (see discussion above). If the binding preferences to a sufficient number of isoreceptors are known, including positive and negative isoreceptors, computer-based molecular modelling of the isoreceptor sequences

may inform us of steric hindrances and other causes of changed interaction. This allows a conclusion to be drawn as to which side of the minimum common sequence the binding occurs and what effects neighbouring groups may have. A good example is the minimum epitope on isoreceptors of cholera toxin compared with isoreceptors of tetanus toxin (17, and to be published). In Fig. 8 proposed minimum epitopes have been indicated on molecular models of the optimal natural receptors for the two toxins, GM1 and GQ1b gangliosides, respectively. Of interest is the limited minimum requirement found on one side and internally in these molecules, and that the binding epitopes are highly overlapping, indicating a structural and evolutionary relation between the complementary binding sites of the two toxins. The toxins are thus members of the NeuAc-binding family as defined in Table 1. Therefore, probably only a slight change in the amino acid sequence of the receptor-binding site may explain the quite separate target cells (intestine and nerve) producing different symptoms (diarrhoea and tetanus). Theoretically, a synthetic analogue may be designed that will inhibit the binding of both toxins.

PERSPECTIVES

There is at present rapid development in the field of microbial interactions with animal cells based on protein-carbohydrate interactions. Important findings are the internal recognition of the receptor sequence and families of receptor-binding variants. In one single case, the concept of anti-adhesion therapy has been proven, and it is now technically possible to develop this field to encompass broad biomedical applications including therapy of human infections. Globally, there are major infectious diseases such as malaria and AIDS where there is growing evidence that protein-carbohydrate interactions are essential for the establishment of an infection.

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Bacterial Adherence: Adhesin-Receptor Interactions Mediating the Attachment of Bacteria to Mucosal Surfaces

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Recent studies have indicated that the attachment of bacteria to mucosal surfaces is the initial event in the pathogenesis of most infectious diseases due to bacteria in animals and humans. An understanding of the mechanisms of attachment and a definition of the adhesive molecules on the surfaces of bacteria (adhesins) as well as those on host cell membranes (receptors) have suggested new approaches to the prevention of serious bacterial infections: (*1*) application of purified adhesin or receptor materials or their analogues as competitive inhibitors of bacterial adherence; (*2*) administration of sublethal concentrations of antibiotics that suppress the formation and expression of bacterial adhesins; and (*3*) development of vaccines against bacterial surface components involved in adhesion to mucosal surfaces. Progress has already been made in the development of antiadhesive vaccines directed against the fimbrial adhesins of several human bacterial pathogens.

Bacteria stick to and grow on almost any surface [1, 2].

> Within minutes after submerging a solid object in seawater or freshwater, the surfaces become colonized by adherent microorganisms, and the earliest organisms to adhere are bacteria. Adherent colonies of bacteria have also been observed on particles of sand, soil, other bacteria, plant tissues, and a variety of animal tissues. Shortly after birth, the skin and the mucosal surfaces of the upper respiratory tract and the gastrointestinal tract become heavily colonized by a variety of adherent bacteria which persist in varying numbers as indigenous parasites. The apparent symbiotic balance between the host and his indigenous parasites occasionally is upset by the invasion of harmful bacteria which adhere to and colonize these surfaces. Pathogenic bacteria may also adhere to and colonize normally sterile surfaces such as the mucosa of the genitourinary tract and the lower respiratory tract, and occasionally even endothelial surfaces of the cardiovascular system, resulting in the development of serious infectious disease. . . . [1]

Although marine microbiologists have been aware for a long time that bacteria must stick to surfaces in order to avoid being swept away by moving streams of water [3], not until recently has it been widely recognized that adherence must be an important ecological determinant in the colonization of specific sites in plants and animals, and, in particular, an important early event in the pathogenesis of bacterial infections in animals and humans [2]. It is true that, as early as 1908, Guyot [4] reported studies on the adhesiveness of bacterial cells for blood erythrocytes, and that some 20 years ago Duguid and Old [5] had already demonstrated the mannose sensitivity of the adherence of several genera and species of gram-negative bacteria to erythrocytes and intestinal epithelial cells. Nevertheless, the study of the mechanisms of bacterial adherence did not really catch on until about 10 years ago, when Gibbons and his colleagues began reporting a series of elegant studies showing the selective nature of the adherence of bacteria to the various niches of the oral cavity and dental surfaces [2, 6]. Largely because of these studies, bacterial adherence has grown into one of the most active, if not the most exciting, areas of study in the field of microbial ecology and infectious diseases.

As is readily apparent from the large number of papers published during the past five years, I shall be unable to cover the whole field of bacterial adherence in any depth. Instead of presenting a superficial view of a great number of different organisms, I have chosen to present in detail the adherence mechanisms of one microorganism, *Streptococcus pyogenes*, which I know the best

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and which illustrates the general principles of interaction between bacterial adhesin and host cell receptor. In addition, I shall summarize relevant adhesin-receptor studies of other microorganisms. I shall examine three main questions. (*1*) Do bacteria adhere to host cells via specific surface molecules of recognition? In other words, is the interaction a specific or a nonspecific phenomenon? (*2*) Is bacterial adherence a prerequisite for bacterial infectivity? Must bacteria always stick to tissue surfaces to cause infections? (*3*) Can bacterial infections be prevented by blocking the adherence of bacteria to mucosal surfaces? Particular attention will be given to present and possible future strategies for the development of vaccines directed against the surface adhesins of bacterial pathogens.

Before we proceed further, several important terms need to be defined. Two of these terms, adhesin and receptor, have already been used above. Adhesins are the adhesive structures on the surfaces of microorganisms, and receptors are complementary adhesive structures on the surfaces of host cells. Adhesin, first coined by Duguid [7], is preferred to the term ligand because the latter usually refers to a small molecule, whereas bacterial adhesive structures often are composed of large polymeric structures, such as the adhesive filamentous appendages of many Enterobacteriaceae. These adhesive appendages shall be called fimbriae [8] rather than pili [9], the latter term being reserved, as suggested by Jones [10] and Duguid and Old [5], for those appendages involved in the conjugative transfer of DNA. Some adhesins, such as the hairy projections on streptococci [11], lack regular size and shape and will be referred to as fibrillae [10].

The discussion in this paper will be limited to those organisms that invade the host primarily through mucosal surfaces. The adherence of bacteria to tooth surfaces and the pathogenesis and immunology of dental caries will be excluded, except for comparative purposes. Gibbons and van Houte [6] reviewed the subject in 1980.

Specificity of the Adherence of Bacteria to Mucosal Surfaces

I shall now address the first question: do bacteria adhere to host cells via specific surface molecules of recognition? For the answer let us look at both in vivo and in vitro evidence.

Tissue tropism. The specificity of the interaction of bacteria with host tissues was first suggested by in vivo evidence. Gibbons [2] has pointed out the apparent preference of particular bacteria for certain tissues over others (tissue tropism). For example, *Streptococcus mutans*, a cariogenic bacterium, was found in abundant numbers in dental plaque but only in sparse numbers or not at all on the epithelial cells of the tongue. The reverse was true for *Streptococcus salivarius*, an α-hemolytic *Streptococcus* normally found in the human oral cavity. Although attached in high numbers to tongue epithelial cells, the *S. salivarius* organisms were absent from dental plaque [6]. Another classic example is the contrast between two pathogens, *S. pyogenes* and *Escherichia coli*. *S. pyogenes* organisms (or group A streptococci) are virtually limited to the nasopharynx and skin of humans [12], whereas *E. coli* organisms are the most common cause of genitourinary tract infections and rarely colonize the nasopharyngeal cavity. There are exceptions, of course, as pointed out by Johanson et al. [13, 14]; hospitalized patients who have underlying diseases often become colonized in their upper respiratory tracts by *E. coli* and other gram-negative bacteria.

Species specificity. The idea of the specificity of the interaction between bacteria and host tissue is further supported by the species specificity of certain bacterial infections. For example, gonococcal infections are limited to humans [15]; diarrheagenic *E. coli* K88 infections, to pigs [16]; and *E. coli* CFA/I and CFA/II (colonization factor antigens) [17] and group A streptococcal infections [12], to humans. Cheney et al. [18] demonstrated rather convincingly the high degree of species specificity of a diarrheagenic strain of *E. coli* that was able to produce diarrhea in rabbits but not guinea pigs and rats. Moreover, the inability to produce diarrhea in guinea pigs and rats was associated with a much shorter interval of excretion of *E. coli* organisms by the animals fed these bacteria and with the inability of isolated intestinal epithelial cells from these resistant animals, as well as from humans, to bind the microorganisms in vitro. This behavior contrasted with the avid adherence of the microorganisms to brush borders of intestinal epithelial cells isolated from the susceptible rabbit species [18].

Genetic specificity. Perhaps even more convincing has been the evidence that the susceptibility to certain infections is a genetic trait. Sellwood et al. [19] have shown, for example, that certain

pigs are genetically immune to *E. coli* K88 infections. By cross-breeding susceptible and resistant pigs, these researchers were able to show that susceptibility is coded for by autosomal dominant genes and, moreover, is mediated by receptors for *E. coli* K88 on the brush borders of the intestinal epithelial cells.

Miller et al. [20] have presented considerable evidence that susceptibility to *Plasmodium vivax* infestations is dependent on the presence of the Duffy antigen on the surfaces of the host's red blood cells. Black Africans who lack the Duffy antigen are genetically immune to these malarial infestations. It remains to be established, however, whether the Duffy antigen serves as the receptor for the attachment of the organisms to the red blood cell membranes [21].

These lines of in vivo evidence have pointed to the specificity of the association of microorganisms with host tissues and lend support to the idea that adhesive molecules (or adhesins) on the surfaces of the bacteria are recognized by specific receptor molecules on animal cells. It must be emphasized, however, that the association of bacteria with specific tissues in vivo probably is a complex process. Those of us who have used in vitro systems to study bacterial adherence often have been tempted to overlook some of the complexities of the phenomenon in the intact host. Freter [22] has pointed out, for example, that infections of the gastrointestinal tract by *Vibrio cholerae* organisms are dependent on a number of factors in addition to binding of the bacteria to the intestinal epithelial cells. These factors include bacterial motility, chemotaxis, penetration of the mucous gel on the intestinal villi, adhesion to receptors in the mucous gels, chemotaxis into deeper intervillous spaces, and, finally, attachment to the epithelial cells and elaboration of the injurious cholera toxin. Thus, pathogenic organisms must overcome a number of nonspecific local defenses before they are able to attach to the epithelial cells.

Nonspecific factors influencing bacterial adherence. The mucosal surfaces of the healthy host are usually impervious to pathogenic organisms because of a number of cleansing mechanisms that operate on these surfaces. Mucosal surfaces, during good health, are constantly bathed by secretions laden with antibacterial enzymes and antibodies, which impede the attempts of pathogens to colonize the surfaces. The unattached organisms are then simply swept away in the luminal contents by mechanical means such as coughing, sneezing, ciliary action, swallowing, peristalsis, and excretion. Pathogenic organisms are able to gain a foothold only by taking advantage of impaired local defense mechanisms. Even then, organisms that are able to attach may be eliminated by desquamation of the colonized epithelial cells. Successful pathogens are those capable not only of penetrating the local defenses and attaching to mucosal cells, but also of replenishing the new surfaces as colonized cells are desquamated and, eventually, of penetrating the epithelial cell barrier by invasion, either by the organism itself or by an excreted toxin [23].

It is obvious, then, that one must be cautious in interpreting the in vivo significance of data obtained from in vitro experiments. With this caution in mind, however, in vitro experiments have proved useful in defining the adhesive structures that bind the bacteria to host cells.

The virtual irreversibility of binding has indicated that, almost certainly, the bacteria are held in place by many independent bonds between the bacteria and host cell surfaces [24]. Several nonspecific factors are important determinants in the formation of multiple bonds between bacteria and host cells. The net charge on the surface of both the bacterial cells and the host cells is negative, creating repulsive forces between the cells. These repulsive forces, however, may be overcome by the attractive forces between hydrophobic molecules present in varying numbers on the two cell surfaces. It should be noted also that two surfaces, although negatively charged, may nevertheless attract each other by long-range forces created by atomic and molecular vibrations that produce fluctuating dipoles of similar frequencies on each surface (the DLVO theory [10]).

Specific binding. Once the nonspecific attractive forces overcome the repulsive forces, the organism is allowed to approach the host cell surface and to "dock" [25] in a rather loose, reversible fashion. Permanent anchoring to the surface requires the formation, then, of many specific lock-and-key bonds between complementary molecules on each cell surface, as is shown diagrammatically in figure 1. The complementary receptor and adhesin molecules must be accessible and arranged in such a fashion that many bonds may form over the area of contact between the cells. Once these bonds are formed, attachment under physiological conditions becomes virtually irreversible because,

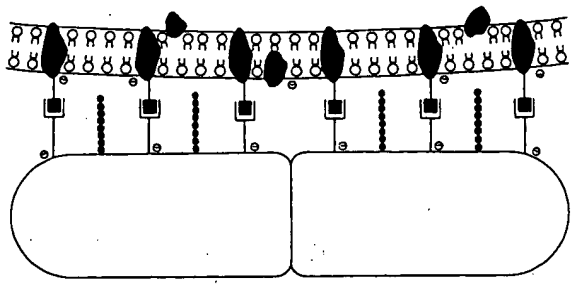

Figure 1. Attachment of bacterial cells (*bottom*) via specific adhesins (fork-like structures) to complementary receptors (■) on the host cell membrane (*top*). To overcome the net negative charge (⊖) on both the bacterial and the host cell surfaces, hydrophobic molecules (regular black structures shown inserted in the host cell membrane) are attracted toward the hydrophobic phospholipid molecules (circles with legs) in the lipid bilayer membrane. The irregular black structures represent protein or glycoprotein incorporated into the host cell membrane. Reproduced by permission from Ofek and Beachey [23].

even if each bond, by itself, was relatively weak, the likelihood that all of the bonds would be broken at the same time would be very small [24].

How do we go about proving that specific receptor and adhesin molecules mediate the binding of a particular species of bacteria to host cells? Indirect evidence would include showing that: (*1*) adherence of the bacteria to tissue cells is inhibited by adhesin or receptor analogues, by enzymes and chemicals that specifically destroy adhesins and receptors, and by antibodies to specific surface components; and (*2*) the bacteria can bind to receptor analogues. Direct evidence would include isolation and purification of the bacterial adhesins and host cell receptors as well as showing that the isolated receptor binds to specific sites on the bacterial surface, whereas the isolated adhesin binds to specific receptors on the surfaces of epithelial cells. The isolated materials, of course, must be able to block bacterial adherence, as is shown diagrammatically in figure 2.

Adherence Mechanisms of Group A Streptococci

I shall now show the application of the principles outlined above to the study of adherence mechanisms of group A streptococci (*S. pyogenes*). When suspensions of group A streptococci are mixed with scrapings of pharyngeal epithelial cells, the bacteria adhere to certain areas but not to others on the surface of the cell (figure 3), an observation suggesting that receptors for the attachment of the organisms have a patchy distribution in the host cell membrane. We shall first examine the evidence for the existence of a specific adhesin that binds to the epithelial cell receptors.

The streptococcal adhesin. Ofek and I examined various surface components of group A streptococci as possible candidates for the adhesin [27, 28]. Of the components tested, which included lipoteichoic acid (LTA), M protein, C carbohydrate, and a sonicate of peptidoglycan, only LTA was able to block adherence to any significant degree. Because LTA was known to be an amphipathic molecule, lipopolysaccharides isolated from *E. coli* and *Serratia marcescens* were also tested; they were found to have no inhibitory effect, a result suggesting specificity of the inhibitory effect of LTA. Specificity was further shown by the in-

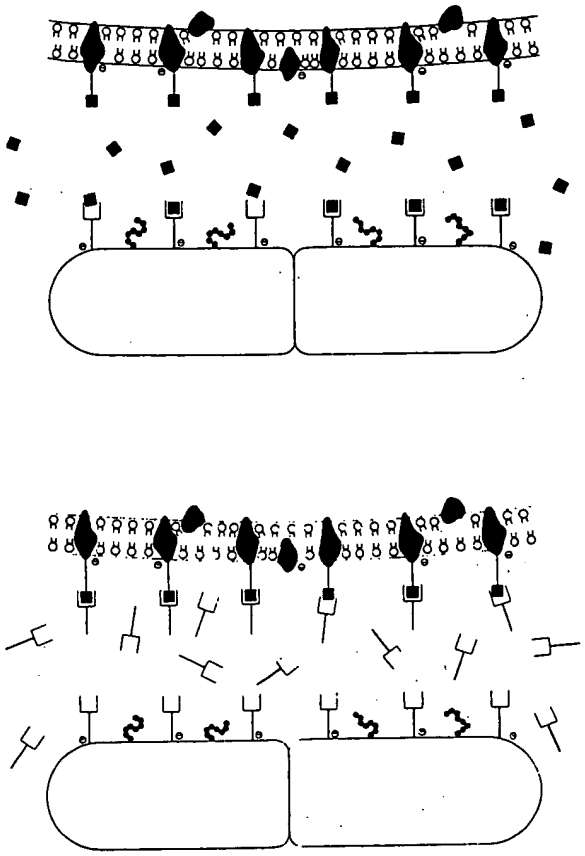

Figure 2. Specific blockade of bacterial adherence by an excess of (*top*) isolated receptor analogue material (■) or (*bottom*) isolated adhesin or adhesin analogue material (fork-like structures). Reproduced by permission from Ofek and Beachey [23].

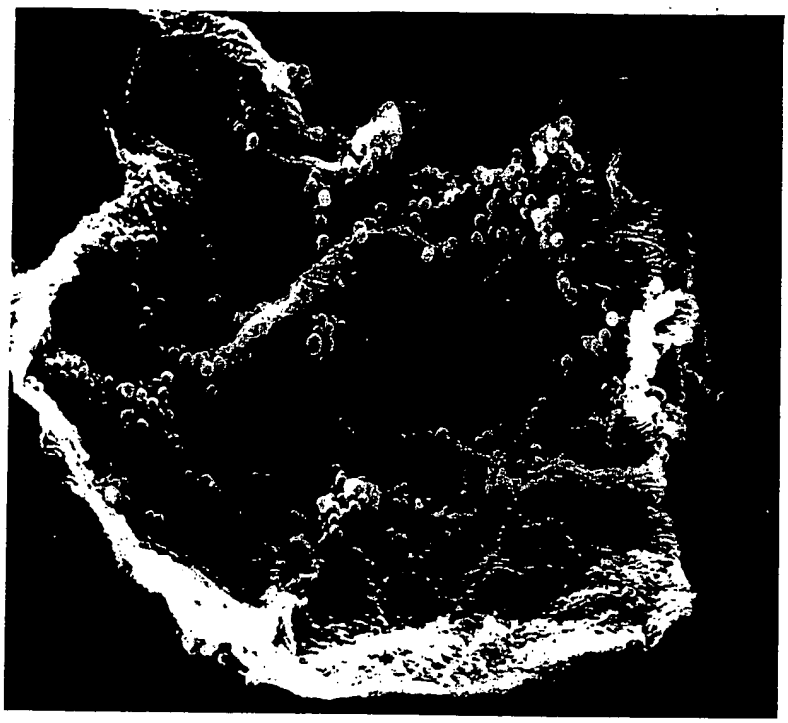

Figure 3. Scanning electron micrograph of *Streptococcus pyogenes* organisms attached in patches to an isolated human pharyngeal epithelial cell (courtesy of D. S. Selinger, Albuquerque, N. Mex.). Reproduced by permission from Beachey et al. [26] (×2,000).

ability of LTA to block the adherence of *E. coli*.[1]

As a corollary, treatment of streptococci with diluted antiserum to LTA, but not that to group substance or M proteins, blocked binding of streptococci to epithelial cells. Moreover, absorption of the antiserum to LTA with erythrocytes coated with LTA abolished the inhibitory effect of the antiserum [27, 28].

To determine whether LTA recognized specific binding sites on the surfaces of the host cells, the LTA was labeled by growing cultures with [^{3}H]glycerol. The radiolabeled LTA was isolated, purified, and tested for binding to isolated erythrocyte membranes [29]. We found that binding was dependent on membrane concentration and time (it reached equilibrium at 30 min). The binding sites on the erythrocyte membranes were saturable. Analysis by a Scatchard plot [30] of the binding at several different concentrations of [^{3}H]LTA revealed a single population of binding sites with a dissociation constant of 42 μM (figure 4). Similar specificity of binding has now been demonstrated for platelets [31], lymphocytes [32], intact erythrocytes [33], and oral epithelial cells [34]. These results suggested that host cell membranes contain specific binding sites (or receptors) on their surfaces for streptococcal LTA.

Further evidence of the specificity of binding was obtained from studies of the binding of radiolabeled LTA to resealed right-side-out and inside-out erythrocyte membrane ghosts [29]. We found that the right-side-out ghosts bound 10 times more LTA than their inside-out counterparts (table 1); this observation suggests that specific binding sites for LTA were present on the outer, but not the inner, surface of human cell membranes.

It was of interest, next, to determine whether polyglycerophosphate (PGP) or lipid was the binding moiety of the LTA molecule. For this purpose LTA was cleaved by mild ammonia hydrolysis into a chloroform-methanol–soluble fatty acid fraction and a water-soluble PGP fraction [28, 37]. Our finding that the fatty acid fraction, but not the PGP fraction, inhibited binding of streptococci indicated that the LTA became attached to the cell membrane via its lipid moiety.

The latter finding left us with a dilemma because, according to the model proposed by Wicken and Knox [38], the lipid end of the LTA molecule is anchored by intercalation into the bacterial cytoplasmic membrane, whereas the PGP backbone

[1] H. Courtney, I. Ofek, W. A. Simpson, and E. H. Beachey, "Characterization of the Binding of Lipoteichoic Acid to Polymorphonuclear Leukocytes of Human Blood," manuscript in preparation.

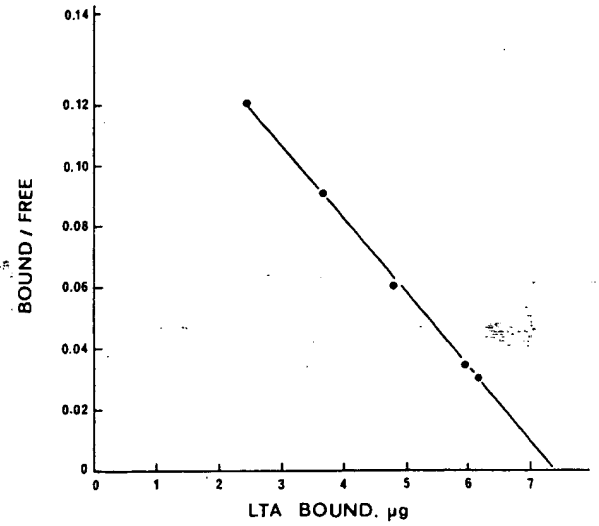

Figure 4. Scatchard plot [30] of the binding of radiolabeled lipoteichoic acid (LTA) to isolated human erythrocyte membranes. The membrane preparations (100 μg of protein) were mixed with 40–200 μg of radiolabeled LTA in a total volume of 100 μl and incubated for 30 min at 37 C. The radioactivity associated with the cell membranes was then assayed. The generation of a linear plot indicates a single population of binding sites. The intercept at the abscissa (7.4 μg) corresponds to an approximation of the total number of binding sites at saturation. Reproduced by permission from Chiang et al. [29].

protrudes outward through the cell wall. If this is so, how can the lipid end of the LTA molecule bind to the cytoplasmic membrane of the bacteria and at the same time serve to bind the organisms to host cells? The answer to this question may lie in the observation that LTA and its deacylated derivative are constantly secreted into the surrounding medium by viable group A streptococci [39]. Thus, the LTA molecule is in constant transit through the cell wall, and, because of its polyanionic character, the PGP end of the molecule may form complexes with positively charged substances in the bacterial cell wall.

To test the possibility, Ofek et al. mixed purified LTA with purified preparations of streptococcal M protein and found that LTA was capable of forming soluble complexes at neutral pH and insoluble complexes at pH 3.7 with each M protein preparation tested.[2] Furthermore, the fact that the complexes were formed equally as well by deacylated LTA indicates that LTA binds to the protein through its PGP backbone. Knowing most of the amino acid sequence of at least one M protein [40, 41] allowed us to show the alignment of the polyanionic backbone of LTA with clusters of positive charges on the M protein molecule.[3] Blocking of the positive charges on the M protein molecule with maleic anhydride blocked the ability to form complexes with LTA without abolishing antigenicity.[4]

The binding between LTA and surface protein would anchor the LTA molecule to the bacterial surfaces and leave the lipid end free to interact with LTA receptors on the host cell membranes. Putting this information all together into what Ofek and I have called a "tree theory," we believe the LTA and deacylated LTA form a complex network with the M protein and other LTA-binding proteins on the surface of the *Streptococcus* (figure 5). According to this theory, LTA and LTA-binding proteins anchor each other to the surface of the *Streptococcus*. These complexes allow the lipid ends of some LTA molecules to be intercalated into the cytoplasmic membranes of the bacterium, while others are free to interact with LTA receptors on host cell membranes. The idea that lipid moieties are exposed on the surfaces of streptococci is consistent with the recent findings of Tylewska et al. [42] that the surfaces of group A streptococci are highly hydrophobic. This is not surprising in view of the earlier findings by Hill et al. [43] that the surfaces of these organisms are rich in fatty acids. We now believe the LTA-protein complexes may compose the fibrillae on the surfaces of the streptococci. As seen by electron microscopy, these structures seem to be involved in the binding of the organisms to epithelial cells (figure 6). Fatty acid ends of LTA molecules exposed at the outer end of the fibrillae may interact with specific receptors in the cell membrane.

Membrane receptors for LTA. We would have liked to isolate a material from host cell membranes that would inhibit the attachment of streptococci. Unfortunately, such receptor material is not yet available. Therefore, we turned to a search for analogues of the putative LTA receptor. Having established that LTA binds via its lipid end to cell membranes, we realized that the well-known fatty acid binding sites on serum al-

[2] I. Ofek, W. A. Simpson, and E. H. Beachey, "Orientation and Anchorage of Lipoteichoic Acid on the Surface of *Streptococcus pyogenes* Cells," manuscript in preparation.

[3] See footnote 2.

[4] See footnote 2.

Table 1. Preferential binding of lipoteichoic acid (LTA) to the outer surface of human erythrocyte membranes.

Membrane preparation	G3PD activity (units/mg)*	Acetylcholinesterase activity (units/mg)†	LTA binding (cpm/mg of membrane protein)‡
Unsealed ghosts	0.266	1.15	3,960
Resealed ghosts			
Right-side-out	0.072	1.07	3,800
Inside-out	0.635	0.13	300

* The activity of glucose-3-phosphate dehydrogenase (G3PD), a marker for the inner surface of the erythrocyte membranes [35], was assayed by a modification of the method of Krebs [36], as described by Chiang et al. [29].

† The activity of acetylcholinesterase, a marker for the outer surface of the erythrocyte membrane, was assayed by the method of Steck and Kant [35], as described by Chiang et al. [29].

‡ The membrane preparations (100 μg of protein) were mixed with 40 μg of [³H]LTA in a total volume of 100 μl and incubated for 30 min at 37 C. The [³H]LTA that formed complexes with the membrane was separated from the unbound [³H]LTA by membrane filtration under reduced pressure and assayed for radioactivity [10].

bumin might serve as an excellent receptor analogue.

In the initial experiments, Simpson et al. found that albumin blocked the binding of streptococci in a dose-response fashion [44, 45]. Moreover, the inhibition was specific for streptococci; the binding of an *E. coli* strain that binds to mannose residues on cell membranes [5, 46] was not affected by albumin.[5] Conversely, α-methylmannoside, which blocked the binding of *E. coli*, had no effect on the binding of the streptococcal cells. Absorption of the inhibitory effects of albumin solutions by streptococci, but not epithelial cells, indicated that inhibition was due to binding of the albumin to the surface of the streptococci. The binding of albumin to the surface of streptococci has recently been reported by Kronvall et al. [47] and Myhre and Kronvall [48].

To prove further that LTA became bound to albumin, Simpson et al. showed that LTA was able to protect the albumin from heat denaturation and to form complexes that could be demonstrated by immunoelectrophoresis [44]. Furthermore, albumin blocked the binding of radiolabeled LTA to human erythrocytes in a dose-related fashion (figure 7, left). The binding of increasing concentrations of LTA to erythrocytes was tested in the absence and in the presence of two different concentrations of albumin, and the data were analyzed in a double reciprocal plot (figure 7, right). The convergence of all the lines to one point on the ordinate indicated that albumin and the cell membrane receptor bound to the same site on the LTA molecule [45].

The final question was whether albumin would be able to bind to LTA that had formed complexes with M protein. Such binding would lend support to the idea that the lipid moiety of the LTA molecule remains free to react with membrane receptor molecules. To test this possibility, Ofek et al.

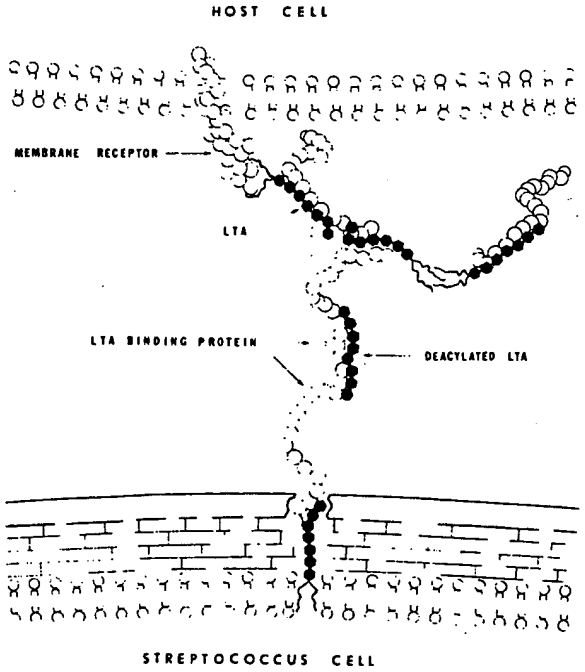

Figure 5. Diagrammatic representation of the interaction between lipoteichoic acid (LTA) and M protein on the surface of *Streptococcus pyogenes* cells (*bottom*). The cross-linking by LTA and deacylated LTA of several LTA-binding protein molecules would result in the formation of a fibrillar network of LTA and protein and would permit the exposed lipid ends of firmly anchored LTA molecules to interact with receptors in host cell membranes (*top*). A micromicelle formed between two LTA molecules is shown at upper right. Reproduced by permission from Beachey et al. [26].

[5] See footnote 2.

Figure 6. Electron micrograph of an ultrathin section of a *Streptococcus pyogenes* organism (*center*) adherent to pharyngeal epithelial cells (E). The surface fibrillar network, composed of lipoteichoic acid-protein complexes, radiates from the surface of the *Streptococcus* to the epithelial cell membrane (arrows). Reproduced by permission from Beachey and Ofek [27].

prepared insoluble complexes at pH 3.7 between M protein and LTA or deacylated LTA.[6] Once formed, the complexes remained stable even at neutral pH, and the complexes formed between M protein and LTA bound 10 times more albumin than the complexes formed between M protein and deacylated LTA, a result that supports the idea that the lipid end of the LTA-M protein molecule remains free to interact with receptor molecules (figure 5).

In summary, the evidence that LTA serves as an adhesin for the adherence of group A streptococci is as follows. (*1*) LTA, but not other surface components, inhibited adherence of streptococci but not adherence of *E. coli*. (*2*) Diluted antiserum to LTA, but not antisera to other surface components, inhibited adherence. (*3*) LTA bound to a single population of binding sites in host cell membranes. (*4*) LTA bound to right-side-out but not to inside-out erythrocyte membrane vesicles.

The evidence that serum albumin is a receptor analogue for the binding of streptococci to host cells is as follows. (*1*) Albumin inhibited adherence of streptococci but not *E. coli* to epithelial cells. (*2*) Albumin bound to the surface of streptococcal cells. (*3*) LTA bound to the fatty acid binding sites of albumin. (*4*) Albumin competitively inhibited the binding of LTA to host cells.

We still have no data on an isolated cell membrane receptor for LTA, but the putative receptor very well may be a membrane protein or glycoprotein that bears fatty acid binding sites similar to those on albumin.

[6] See footnote 2.

Adherence Mechanisms of Other Bacteria

By applying the principles outlined above, the adhesins and receptors for a number of other bacterial species have now been definitely or tentatively identified and are summarized in table 2. In general, the adhesins are associated with surface fimbriae in gram-negative bacteria and with the surface fibrillae of gram-positive organisms. The receptor-binding site of gonococcal fimbriae has now been localized to a peptide fragment derived by cyanogen-bromide cleavage of the fimbrial subunits [96]. Moreover, antibodies to the peptide containing the receptor-binding domain blocked the adherence of the intact fimbriae to erythrocyte membranes (G. Schoolnik, personal communication). Although a specific structure has, as yet, not been identified for the mycoplasmas, the adhesins appear to be protein in nature.

The receptors on cell membranes for the gram-negative bacteria are in general composed of carbohydrates. In some instances—for example, many species of Enterobacteriaceae—the receptor appears to reside in a single sugar, namely, mannose. That is, mannose alone of the many sugars tested is capable of blocking the binding of these organisms to host cells [5, 46]. In other instances, however, the receptors appear to be composed of more complex carbohydrate polymers. Examples of the latter are the uropathogenic and the diarrheagenic *E. coli* as well as the gonococci. Finally, as shown by the data in the present paper, the receptor for the most thoroughly studied gram-positive organism, *S. pyogenes*, appears to reside in an albumin-like membrane protein or glycoprotein (table 2).

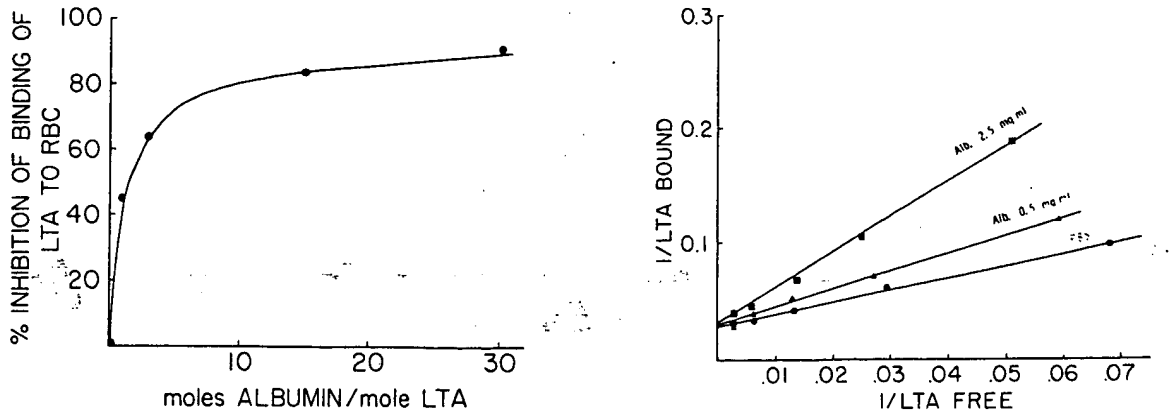

Figure 7. *Left*, inhibition of the binding of radiolabeled lipoteichoic acid (LTA) to human erythrocytes (RBC) by bovine serum albumin. Reproduced by permission from Simpson et al. [45]. *Right*, double reciprocal plot of the inhibition of LTA binding to human erythrocytes by bovine serum albumin (Alb). The convergence of all three lines at one point on the ordinate indicates competitive inhibition between albumin and the putative membrane receptor for LTA [45]. PBS = phosphate-buffered saline.

In addition to the native receptors for bacteria on host cells, there is now considerable evidence that new receptors for bacteria may be formed by virus-infected epithelial cells [97–101]. This appears to be especially true for cells infected with influenza virus. It has been shown that influenza virus infection of laboratory animals predisposes them to infections by a variety of bacterial species. Sanford et al. [98–100] and Elbein et al. [101] have shown that certain strains of group B streptococci and *Streptococcus sanguis* adhere to influenza virus–infected canine kidney epithelial cells but not to uninfected cells. Moreover, binding to the virus-infected cells was blocked by preincubating the streptococci with free virus particles [101]. Further studies [101] using protein glycosylation inhibitors indicated that the streptococci were recognizing specific viral glycoproteins produced in the cell membranes of virus-infected cells. Ramphal et al. [102] have shown that *Pseudomonas aeruginosa* organisms adhered more avidly to mouse tracheal epithelial cells when the cells were infected with influenza virus, although the mechanism of the increased adherence remains unclear. Nevertheless, these studies indicate that, in addition to native receptors on host cells for pathogenic bacteria, new receptors may be generated in virus-infected tissues.

Bacterial Adherence vs. Bacterial Infectivity

On the basis of the foregoing evidence, it can be concluded that specific molecules of recognition exist between various species of pathogenic bacteria and the tissues of the host. This conclusion, then, leads to the second question: is bacterial adherence a prerequisite for bacterial infectivity? The question may be approached in two ways. First, one can examine the relationship between the ability of particular species of pathogenic bacteria to adhere to epithelial cells in vitro and their ability to produce infectious diseases. Second, one can examine the ability of epithelial cells isolated from infection-prone patients to bind bacteria.

Relationship between adherence in vitro and infectious diseases. Examples of bacteria whose adherence has been studied and compared with the relative infectivity of variants of the organisms in vivo are listed in table 3. In general, infectivity paralleled adhering ability except for *Salmonella*, which, although poorly adherent in vitro, was moderately infective. Of particular interest were the findings of Satterwhite et al. [105], who isolated one colonization factor–negative mutant from an enterotoxigenic strain of *E. coli* and showed that, even though this nonadherent isogenic mutant retained its ability to produce enterotoxin, it was unable to produce diarrhea in human volunteers. Furthermore, the nonadherent derivative was shed in the stools of infected volunteers for much shorter intervals than was the parent adherent strain. These studies strongly suggested that the bacteria must be able to attach to the intestinal epithelial cells in order to deliver the diarrhea-producing toxins effectively.

Table 2. Specific adhesins and receptors of various bacteria.

Bacteria	Adhesin	Receptor*	Investigators
Streptococcus pyogenes	Lipoteichoic acid–M protein fibrillae	Albumin-like protein?	Ofek et al. [37]; Beachey and Ofek [27]; Beachey [28]; Simpson et al. [44, 45]
Escherichia coli; Klebsiella; Serratia; Shigella; Enterobacter; Salmonella; Citrobacter	Type I fimbriae	D-mannose	Duguid et al. [8]; Duguid and Old [5]; Ofek et al. [46]; Salit and Gotschlich [49, 50]
E. coli (uropathogenic)	Fimbriae	GalNacα1-3GalNacβ1-3Galα1-4Galβ1-4GlcCer (globotetraosylceramide)	Svanborg-Eden [51]; Svanborg-Eden and Hansson [52]; Korhonen et al. [53]; Leffler and Svanborg-Eden [54]
E. coli CFA/I and CFA/II†	Fimbriae	?GalNacβ1-4Galα1-4GlcCer (GM_1 ganglioside)	McNeish et al. [55]; Evans et al. [56–58]; Ørskov and Ørskov [59]; Evans and Evans [60]; Faris et al. [61]
E. coli K88	Fimbriae	?β-D-Gal or GalNac and GlcNac	Stirm et al. [62]; Jones and Rutter [16, 63]; Wilson and Hohmann [64]; Hohmann and Wilson [65]; McNeish et al. [55]; Nagy et al. [66]; Gibbons et al. [67]; Anderson et al. [68]
E. coli K99 and 987	Fimbriae	?GalNacβ1-4Galβ1-4GlcCer	Ørskov et al. [69]; Burrows et al. [70]; Isaacson [71]; Faris et al. [61]; van Embden et al. [72]
Vibrio cholerae	Fimbriae	Fucose and mannose	Tweedy et al. [73]; Jones and Freter [74]; Nelson et al. [75]; Jones [76]
Mycoplasma	Membrane protein	Sialic acid; glycophorin	Sobeslavsky et al. [77]; Banai et al. [78, 79]; Hu et al. [80]; Gorski and Bredt [81]; Gabridge et al. [82, 83]; Gabridge and Taylor-Robinson [84]; Feldner et al. [85]
Neisseria gonorrhoeae	Fimbriae	Galβ1-3GalNacβ1-4Gal	Pearce and Buchanan [86]; Watt and Ward [87]; Punsalang and Sawyer [88]
Proteus	Fimbriae	Not known	Duguid and Gillies [89]; Shedden [90]; Duguid and Old [5]; Silverblatt [91]; Silverblatt and Ofek [92]
Bordetella pertussis	Fimbriae	Possibly sterol	Sato et al. [93]; Morse and Morse [94]
Pseudomonas aeruginosa	Fimbriae	Not known	Woods et al. [95]

* Gal = galactose; GalNac = *N*-acetylgalactosamine; Glc = glucose; Cer = ceramide.
† CFA = colonization factor antigen.

The advantage that intimate association with host cells affords for toxin-producing *E. coli* is illustrated diagrammatically in figure 8. The organisms that become attached to intestinal epithelial cells are able to deliver their toxin molecules in higher concentrations to the toxin receptors in the cell membranes. The close association also prevents loss of toxin activity due to degradation by enzymes and chemicals in the extracellular and mucosal fluids. A similar advantage of attachment was described by Sobeslavsky et al. [77] for *Mycoplasma pneumoniae* organisms. Attachment to tracheal epithelial cells allows the toxic peroxides produced by these organisms to be transferred directly to the host cell membrane and thereby to bypass the catalase and peroxidase enzymes present in the extracellular fluid and mucosal secretions.

Bacteria-binding capacity of epithelial cells from infection-prone hosts. It has been observed that epithelial cells from certain subjects have an increased capacity to bind pathogenic bacteria. For example, Fowler and Stamey [109], Kallenius and Winberg [110], and Svanborg-Edén and Jodal [111] all have shown that periurethral epithelial cells of females who have experienced recurrent

Table 3. Relationship between adherence of bacteria to epithelial cells in vitro and bacterial infectivity in vivo.

Bacteria	Bacterial variants	Relative adherence in vitro	Relative infectivity in vivo	Investigators
Gonococci	T1 (fimbriated)	Good	High	Swanson [103]; Buchanan and Pearce [104]; Watt and Ward [87]; Pearce and Buchanan [86]
	T4 (nonfimbriated)	Poor	Low	
Escherichia coli (enterotoxigenic)	CF positive	Good	High	Satterwhite et al. [105]
	CF negative	Poor	Low	
Streptococci	Dextran positive	Good	High	Scheld et al. [106]; Ramirez-Ronda [107]
	Dextran negative	Poor	Low	
Salmonella	Fimbriated	Good	High	Duguid and Old [5]
	Nonfimbriated	Poor	Moderate	
E. coli	K88 positive	Good	High	Jones and Rutter [16]
	K88 negative	Poor	Low	
Proteus mirabilis	Fimbriated	Good	High	Silverblatt [91]; Silverblatt and Ofek [92]
	Nonfimbriated	Poor	Low	
Bordetella pertussis	Fimbriated	Good	High	Sato et al. [93]
	Nonfimbriated	Poor	Low	

urinary tract infections bind a higher number of *E. coli* cells than do periurethral epithelial cells from females who are not prone to such infections. Aly et al. [112] have shown that nasal epithelial cells from staphylococcal carriers bind considerably more *Staphylococcus aureus* than do nasal cells from noncarriers. Ofek et al. have observed a decreased ability of oral epithelial cells from newborn infants to bind streptococci, and the decreased binding capacity was associated with a sparsity of bacteria in the oral cavity for the first few days of life [113]. Perhaps even more convincing is the *E. coli* K88 story. The brush borders of epithelial cells from piglets resistant to diarrhea produced by *E. coli* K88 fail to bind the organisms, whereas the brush borders of susceptible piglets bind the organisms in high numbers [19]. As I have previously mentioned in this paper, crossbreeding of resistant and susceptible pigs has shown that both the binding ability and the susceptibility to diarrhea are autosomal dominant traits [19]. Therefore, pigs that lack receptors for *E. coli* K88 in the brush borders of their epithelial cells can be considered to be genetically immune.

Thus, although the evidence is still indirect, these studies point toward the importance of adhesion of microorganisms to epithelial cells as an essential first step in the pathogenesis of infectious diseases. One can assume that adhesion is advantageous, first, for the survival of the pathogen

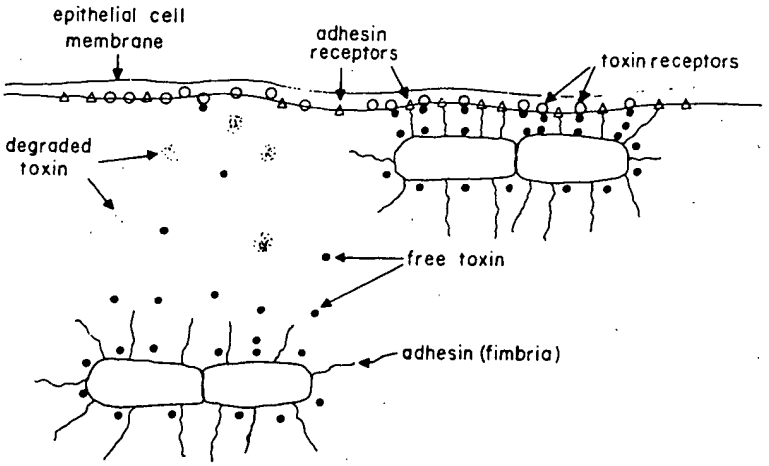

Figure 8. Advantage of bacterial adherence for efficient delivery of toxin to membrane receptors of host cells. A similar model has been proposed by Middeldorp and Witholt [108] for the delivery of the heat-labile toxin of a K88 strain of *Escherichia coli* to microvillar membranes of intestinal cells of the pig.

and, second, for the efficient delivery of its injurious toxins to susceptible tissues. It must be pointed out, however, that adhesion may not always be advantageous for the bacteria.

Good vs. bad adherence. Silverblatt [91] and Silverblatt and Ofek [92, 114] have demonstrated that, whereas fimbriated *Proteus mirabilis* adhered readily to urinary tract epithelial cells and were able to cause ascending pyelonephritis in rats, the same bacteria also attached more readily than their nonfimbriated counterparts to phagocytic cells. Once attached, the heavily fimbriated bacteria were rapidly ingested and killed. These relationships were demonstrated by in vivo experiments in which heavily fimbriated or lightly fimbriated bacteria were introduced either intravesicularly or iv. The researchers found that the fimbriated organisms were readily able to produce ascending pyelonephritis but were unable to infect the kidney when they were injected iv. Lightly fimbriated bacteria that adhered poorly to epithelial cells did not produce pyelonephritis by the ascending route but were able to do so by the hematogenous route. The ease of producing ascending infections paralleled the ability of the organisms to adhere to isolated epithelial cells, whereas the ease of producing hematogenous infections paralleled the ability of the organisms to resist phagocytosis.

Therefore, in order to survive, not only must the bacteria be able to adhere to mucosal surfaces, they must also be able to adapt to the pressures of the particular microenvironments into which they migrate. In the renal pelvis the bacteria need a high degree of adhering ability to protect themselves against the cleansing effects of mucosal secretions and urine flow. When they invade deeper tissue, however, a high degree of adhering ability would prove suicidal because attachment to phagocytes migrating into the area would result in the engulfment and eventual destruction of the bacteria.

It is probable that pathogenic bacteria adapt similarly during the infectious process in humans. It is well known, for example, that the polysaccharide capsules of *S. pyogenes* and *Streptococcus pneumoniae* are important determinants of virulence. It is thought that the capsules serve an antiphagocytic function because encapsulated organisms evade ingestion and killing by phagocytic cells in the absence of specific opsonic antibodies. Whitnack et al. clearly demonstrated that the hyaluronate capsule of *S. pyogenes* interfered with the attachment step of phagocytosis [115]. On mucosal surfaces, attachment of the organisms to epithelial cells is vital for their survival. However, fully encapsulated pneumococci [116] and streptococci [117] have been shown to adhere very poorly to human pharyngeal epithelial cells. Similarly, we have found that only after fully encapsulated *S. pyogenes* organisms are treated with hyaluronidase to remove their capsules do they adhere well to oral epithelial cells; immediately upon regeneration of the capsules, adhering capacity is again lost (E. Whitnack and E. H. Beachey, unpublished observations). In addition, Craven and Frasch [118] have found that meningococci isolated from the nasopharynx of chronic carriers adhered well to isolated pharyngeal epithelial cells. In contrast, the same organisms isolated from the blood of patients with meningococcal meningitis adhered poorly or not at all.

Each of the above studies indicates that successful pathogens must be highly adaptable in order to survive and produce disease. On mucosal surfaces, the organisms must possess surface adhesins in order to adhere. Immediately after invading deeper tissues, however, they must either shed their adhesin (for example, uropathogenic *P. mirabilis*) or produce masking capsules (for example, *S. pneumoniae* and *S. pyogenes*) in order to avoid attachment to phagocytic cells. These studies support the concept that not all bacterial adhesiveness is advantageous to the bacteria, nor is it all detrimental to the host.

Prevention of Bacterial Adherence

The final question is, can bacterial infections be prevented by blocking the adherence of bacteria to mucosal surfaces?

One of the goals in the studies of bacterial adherence mechanisms is the eventual development of measures to prevent the adhesion of harmful bacteria to mucosal surfaces before the organisms can produce tissue damage. Now that rather convincing evidence has accumulated to show that bacteria do adhere to host cells by specific molecules of recognition and that adhesion to mucosal surfaces is a prerequisite for bacterial infectivity, it seems reasonable to pursue such approaches. One should bear in mind, however, that adhesion of pathogens to phagocytes is probably beneficial to the host; the preventive agents must not interfere with this interaction.

On the basis of the accumulated evidence presented in the foregoing sections, one could approach the question of antiadhesives in several ways. First, one might apply the isolated and purified bacterial adhesin, membrane receptors, or analogues of these substances as competitive inhibitors of bacterial adherence. Second, one might administer drugs that suppress the formation or expression of bacterial adhesins—such as sublethal doses of antibiotics. Third, one might develop adhesin vaccines to induce the formation of local antibodies that could coat the organisms and thereby prevent adhesion. In the following paragraphs I shall review some promising studies in each of these areas.

Application of receptor analogues. Aronson et al. [119] studied the influence of intravesicular instillation of mannose followed by glucose feeding on urinary tract colonization in mice by a strain of *E. coli* that adhered to host cells in a mannose-sensitive fashion. Of 99 control mice receiving phosphate-buffered saline, 70% were colonized, whereas only 20% of those receiving α-methylmannoside were colonized. Administration of α-methylglucoside to a third group of mice did not alter colonization as compared with that in control animals. Microscopic examination of the bladder mucosa confirmed colonization in the control animals and absence of colonization in the animals receiving α-methylmannoside; the bladder mucosal surfaces of the control mice were heavily colonized by *E. coli*, whereas those of the mannose-treated mice were devoid of adherent bacteria [119].

The results of this study supported the idea that a receptor analogue interferes with microbial adherence and colonization in vivo. A note of caution should be sounded, however, because studies have shown that mannose also inhibits attachment of *E. coli* [120–122] and *Salmonella typhi* [120] organisms to phagocytic cells. Thus, treatment with a freely diffusible competitive inhibitor may have its drawbacks; it may be harmful to the host, especially when organisms have invaded deeper tissues and their elimination depends on phagocytic attachment and engulfment.

Sublethal doses of antibiotics. Recent studies [39, 123–129] have indicated that sublethal doses of antibiotics may alter the ability of certain bacteria to adhere to epithelial cells. Alkan and I studied the effect of subinhibitory concentrations of penicillin on the adhesion of *S. pyogenes* to human oral epithelial cells [39]. We found that, in resting cultures of streptococci, sublethal doses of penicillin induced the organisms to secrete large amounts of LTA, the adhesin that binds the organisms to epithelial cells. The loss of cellular LTA was paralleled by a loss in ability to adhere to epithelial cells (figure 9). In contrast, penicillin had no effect on the adhering ability of resting-phase *E. coli* [123, 124]. In addition, Ofek et al. [124] and Eisenstein et al. [125] have shown that streptomycin, an inhibitor of protein synthesis, similarly had no effect on resting-phase *E. coli* cells. During the growing phase, however, *E. coli* cultures exposed to subminimal inhibitory concentrations of either penicillin or streptomycin lost their ability to adhere [125].

The loss in adhering ability induced by penicillin was related to the formation of filaments and the failure to form surface fimbriae (figure 10); flagella formation appeared to be normal. In most cases, sublethal concentrations of streptomycin were also associated with diminished or absent fimbriation [125, 129]. In one strain of streptomycin-resistant *E. coli*, however, Eisenstein et al. found that streptomycin did not suppress the formation of fimbriae [130]. Furthermore, they showed that these fimbriae, when isolated from the drug-grown bacteria, lacked the ability to agglutinate guinea pig erythrocytes [130]. As little as 3 μg of the fimbriae isolated from the control bacteria agglutinated erythrocytes, whereas up to 500 μg of the drug-grown fimbriae was ineffective.

Therefore, antibiotics, as well as other as yet unidentified chemotherapeutic agents, may have an effect on the infectious process other than killing of bacteria. In some organisms, such as *S. pyogenes*, the antibiotic causes a loss of the adhesin needed by the bacteria to bind to epithelial cells [39] and, as a result, may change surface hydrophobicity [127]. In other organisms, such as *E. coli*, sublethal doses of antibiotics prevent the formation and expression of adhesins. In some cases, the fimbrial organelles are synthesized and expressed in normal numbers but are aberrant in morphology and function. Eisenstein is now investigating the possibility that the aberrant fimbriae formed in the presence of streptomycin may be the result of amino acid substitutions in the fimbrial protein, due to misreading of messenger RNA by ribosomes; such misreading is a well-known effect of streptomycin [131]. Whether such sublethal concentrations of antibiotics intermit-

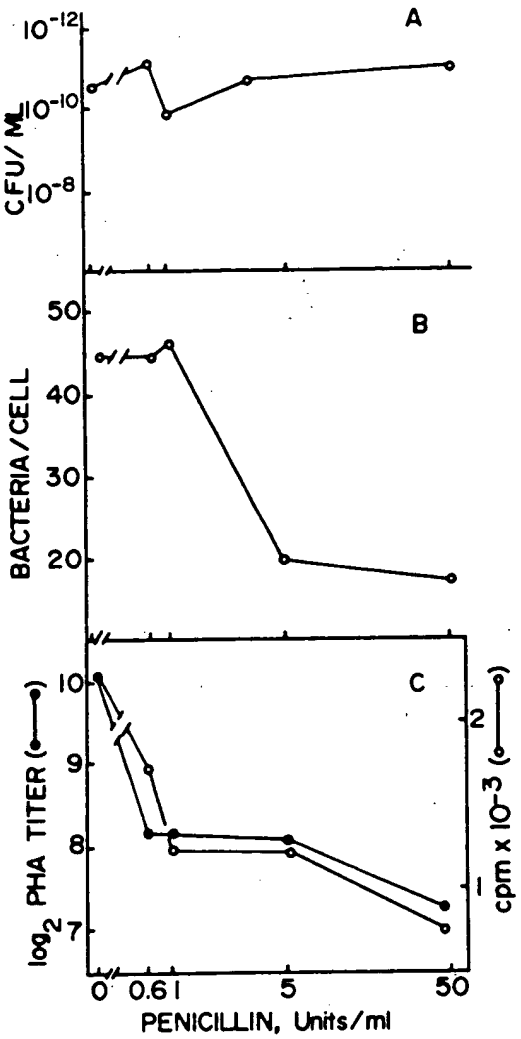

Figure 9. Influence of penicillin on (*A*) viability of streptococcal cells, (*B*) adherence to human oral epithelial cells, and (*C*) amount of lipoteichoic acid (LTA) remaining associated with the bacteria. In *C*, the amount of LTA is expressed as radiolabeled extractable LTA (o——o) and as erythrocyte-sensitizing activity (passive HA [PHA] titer; ●——●). Reproduced by permission from Alkan and Beachey [39].

tently reach the mucosal surfaces during the course of bacterial infections remains to be proved. In any case, in studies of the adhesive mechanisms of bacteria, the use of antibiotics whose mode of action is well known may shed light on the genetics and biochemistry of the adherence of pathogenic bacteria to mucosal surfaces.

Antiadherence vaccines. The ultimate goal in the prevention of bacterial adherence is long-lasting protection. With our present state of knowledge, there is probably no better way to achieve this goal than to rely on immune mechanisms. The ideal candidate for a vaccine against bacterial adherence would be the isolated and purified adhesin itself. Other candidates would include other surface components that are able to evoke antibodies that bind to the surface of the pathogens and sterically block the adhesin from interacting with its receptors on host cells. To be effective, the blocking antibodies must, of course, reach the mucosal surfaces either passively or as secretory antibodies. Thus, antiadhesive vaccines must be able to induce local immunity at susceptible mucosal surfaces.

Several purified fimbrial vaccines prepared from diarrheagenic strains of *E. coli* have been evaluated in farm animals. These include the K88, K99, and 987 fimbriae. The purified vaccines have been administered to pregnant sows and cows in the hope that the offspring would be protected against diarrhea. The premise of these studies has been that the vaccines would evoke antibodies that would be secreted in the mother's colostrum and so be transmitted to the suckling offspring. The results of some of these animal studies are summarized in table 4. Rutter and Jones [132] were able to protect newborn piglets against *E. coli* diarrhea by immunizing the pregnant mothers with doses of up to 45 mg of K88 fimbriae. These researchers obtained brisk humoral immune responses but did not measure mucosal secretory antibodies. Nevertheless, the fact that the offspring were protected suggests the presence of such antibodies in the colostrum. Morgan et al. [133], Nagy et al. [134], and Isaacson et al. [135] immunized pregnant sows with 987 and K99 fimbriae and obtained both humoral and secretory immune responses, with resultant protection of the newborn piglets. Similar immune responses and protection of newborn calves were obtained by Acres et al. [136] by vaccinating pregnant cows with K99 fimbriae. Silverblatt and Cohen [137] vaccinated rats with *E. coli* type I fimbriae (200 μg each). The immunized animals developed humoral antibodies as well as local antibodies (F. J. Silverblatt, personal communication) and were protected against ascending pyelonephritis when challenged with infective doses of *E. coli*. Sato et al. [138] vaccinated mice with the fimbrial hemagglutinin isolated and purified from *Bordetella pertussis* and showed protection against tracheal challenges with the organisms (table 4).

These animal studies hold great promise for

Figure 10. Formation of filaments and lack of fimbriae induced by growth of *Escherichia coli* in subminimal inhibitory concentrations of penicillin. Although the filaments (*bottom*) lacked the fimbriae present on organisms grown without penicillin (*top*), the formation of flagella did not appear to be affected by penicillin.

trials of vaccines in humans against some of the pathogens whose adhesins have been identified (table 5). Recent efforts in several laboratories [139–142] have focused on the development of adhesin vaccines against *Neisseria gonorrhoeae* infections. Purified fimbriae given to human volunteers, in doses ranging from 200 to 2,000 μg, in all cases produced brisk humoral immune responses. Siegal and Buchanan [141] showed that incorporation of certain fimbriae preparations in alum en-

Table 4. Protective bacterial adhesin vaccines in animals.

Adhesin vaccine	Animal	Total antigen dose (mg)	Humoral immune response	Local immune response	Protection against	Investigators
K88 fimbriae	Pregnant sows	45	Yes	Not known	Diarrhea	Rutter and Jones [132]
987 fimbriae	Pregnant sows	10	Yes	Yes	Diarrhea	Morgan et al. [133]; Nagy et al. [134]; Isaacson et al. [135]
K99 fimbriae	Pregnant sows/ cows	18–20	Yes	Yes	Diarrhea	Morgan et al. [133]; Acres et al. [136]; Isaacson et al. [135]
Type I fimbriae	Rats	0.2	Yes	Yes	Ascending pyelonephritis	Silverblatt and Cohen [137]
Fimbrial hemagglutinin	Mice	0.03	Yes	Not known	Pertussis	Sato et al. [93, 138]

Table 5. Protective bacterial adhesin vaccines in humans.

Adhesin vaccine	Total antigen dose (μg)	Humoral immune response	Local immune response	Protection	Investigators
Neisseria gonorrhoeae fimbriae	2,000	Yes	Yes	Yes	Tramont et al. [139]; Boslego et al. [140]
	200	Yes	Yes	Not known	Siegal and Buchanan [141]
Escherichia coli type I fimbriae	45–1,800	Yes	NR*	Equivocal	M. M. Levine, personal communication

* NR = not reported.

hanced immunogenicity. The higher doses of gonococcal fimbriae appeared to afford definite protection in human vaccinees challenged intraurethrally with virulent organisms of *N. gonorrhoeae* [142]. Additional studies are needed to define the antigenic heterogeneity of the gonococcal fimbriae among a large number of pathogenic strains. It is hoped that the receptor-binding domains [96] among the fimbriae from different strains will prove to be antigenically common, and vaccines prepared from peptide fragments containing the common cell-membrane binding region will be broadly protective against many strains of gonococci.

In addition, preliminary clinical trials have been initiated in humans with a type I fimbrial vaccine prepared from an enterotoxigenic strain (H1047) of *E. coli* (table 5). Brisk humoral immune responses were obtained in doses ranging from 45 to 1,800 μg of fimbrial protein. Protection against oral challenges with the vaccine strain of *E. coli* was seen only in the volunteers receiving the higher doses of vaccine. M. M. Levine at the University of Maryland in Baltimore points out that the challenge strain (H1047) of *E. coli* contains both type I and CFA/I fimbriae and that protection in volunteers vaccinated with type I fimbriae may be greater against those enterotoxigenic strains containing only type I fimbrial adhesins (personal communication). Such studies, as well as studies of orally administered type I fimbrial vaccines, are currently being conducted. It would also be desirable, of course, to identify precisely the receptor-binding domain of type I fimbriae, as has been done with gonococcal fimbriae [96], in the hope that such peptide fragments may contain broadly protective immunodeterminants that are readily accessible to the host's immune system.

Although the adhesin for group A streptococci (LTA) has been identified, this molecule is a weak immunogen unless it is complexed to a protein molecule such as methylated bovine serum albumin [27]. Now that we have strong evidence that the LTA molecule forms a complex with M protein or other LTA-binding proteins on the surface of the streptococcal cells, it may be possible to raise adherence-blocking antibodies by immunizing with LTA–M protein complexes. Antibodies to the M protein itself, in sufficiently high titers, may prove to be antiadhesive by sterically blocking the adhesive site on the LTA molecule.

Conclusion

In this paper I set out to answer three basic questions. Do bacteria adhere to host cells via specific surface molecules of recognition? Is the adhesion of bacteria to host cells a prerequisite for infectivity? And, can bacterial infections be prevented by blocking the attachment of bacteria to tissue surfaces? I believe the evidence summarized in this paper is convincing that the answer to each of the three questions is, yes.

Although much work remains to be done, recent advances in defining the molecular basis for the interaction of bacteria with animal cells have provided new leads to the control and treatment of many serious infectious diseases. There is now hope that we shall be able to attack infectious diseases at the first step, before the microorganisms have had the chance to establish themselves and injure the host. Considerable progress has already been made in the development of vaccines directed against the adhesins of several human bacterial pathogens, notably the gonococci and enterotoxigenic *E. coli*. The continued identification and purification of bacterial adhesins and host cell membrane receptors may unravel many of the mysteries of the infectious process and enable us in the near future to eradicate many disabling infectious diseases, as well as their sometimes devastating sequelae.

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Glycoprotein oligosaccharides as recognition structures

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Abstract. A series of observations—the pronounced changes in the expression and distribution of oligosaccharide antigens during embryonic development, cell differentiation and oncogenesis, the prominence of these changing structures (oncodevelopmental antigens) on the receptor for epidermal growth factor, and the stimulation of receptor autophosphorylation following their perturbation with antibodies—has suggested that the oligosaccharides of growth factor receptors and complementary lectins may be intimately involved in molecular recognition events in growth and differentiation processes. For elucidating oligosaccharide recognition by diverse cellular and secreted proteins and microbial adhesins, a new technique has been developed which involves the overlay of immobilized oligosaccharide probes (neoglycolipids) derived from glycoproteins and other sources. New insights have been gained into carbohydrate recognition by several mammalian lectins, and a novel receptor system has been discovered in *Escherichia coli* isolated from patients with urinary tract infections. This new technique seems ideal for elucidating oligosaccharide recognition in diverse biological settings, and for 'quality control' of the sugar chains of recombinant glycoproteins engineered for the purpose of administration to man.

1989 Carbohydrate recognition in cellular function. Wiley, Chichester (Ciba Foundation Symposium 145) p 62–79

With the use of monoclonal antibodies (Feizi 1985, Hakomori 1985, Thorpe et al 1988) and the considerable advances in the techniques of carbohydrate structural analysis (Hounsell 1987, Kobata 1988), we have become increasingly aware of the pronounced changes in the display and positional patterning of various oligosaccharides during embryogenesis and morphogenesis, the continuum of changes in their expression during cell differentiation, and the regular and predictable changes during oncogenesis. Many of the structural changes, particularly those in the backbone and peripheral domains of the oligosaccharides of glycoproteins and glycolipids, are manifest as antigenic changes. Other more recently characterized structures in the backbone and peripheral domains of O-linked oligosaccharides of fetal glycoproteins are

potential antigens (Hounsell et al 1988). Collectively such transiently expressed antigens are referred to as oncodevelopmental antigens (Feizi 1985), many of which are related to the major blood group antigens, A, B, H and Lewis (Watkins 1980, Kabat 1982). All this indicates that there is a remarkable programming of glycosyltransferase activities during the development and differentiation processes. The challenge we now face is to understand the roles of this diversity of structure and the relevance of the changes in the growth and differentiation processes.

Oligosaccharides: ligands in growth regulating networks?

Might oligosaccharides be the missing links in the molecular recognition events in cell growth and differentiation? Two observations have suggested to us that they may be. The first is that one of the major carriers of blood group-related oncodevelopmental antigens is the epidermal growth factor (EGF) receptor (reviewed by Feizi & Childs 1987a). The second is that perturbation of the blood group A-active oligosaccharide chains of the solubilized EGF receptor from A431 cells stimulates receptor autophosphorylation. This conclusion was reached after autophosphorylation experiments by Yarden & Schlessinger (1985) using the solubilized EGF receptor and two monoclonal anti-receptor antibodies which subsequently were shown (Gooi et al 1985) to be directed at the blood group A-related oligosaccharide structures on the receptor glycoprotein. Doubtless, the A431 cell line is derived from a blood group A individual, and alternative oligosaccharide structures would be predicted to occur on EGF receptors from cells lacking blood group A. We believe (Feizi & Childs 1987a) that the importance of these observations is that the reaction of sugar chains of the receptor with complementary antibodies elicited an effect that is associated with receptor activation. Whether this effect is due to receptor aggregation or to allosteric effects on the protein moiety is not yet known. In either case, we interpret the results as being an important clue to the role of oligosaccharides (including the blood group structures in cells that express them normally) in growth regulation. By analogy with the growth-modulating effects of exogenously added plant lectins on animal cells, we proposed that endogenous lectins may react with the sugar chains of growth factor receptors and tune their response to growth factors (Feizi & Childs 1987a). Our thesis is that oligosaccharides of cell surface glycoproteins and glycolipids, and complementary lectins, may constitute networks of functionally coupled receptor systems that transmit signals within and across cell membranes (Fig. 1).

A report by Defize et al (1988) is in strong support of this idea. They have identified and separated two cell populations in A431 cells: the first is blood group A-positive and the second blood group A-negative. These two glycosylation variants differ both in the number of high affinity receptors for

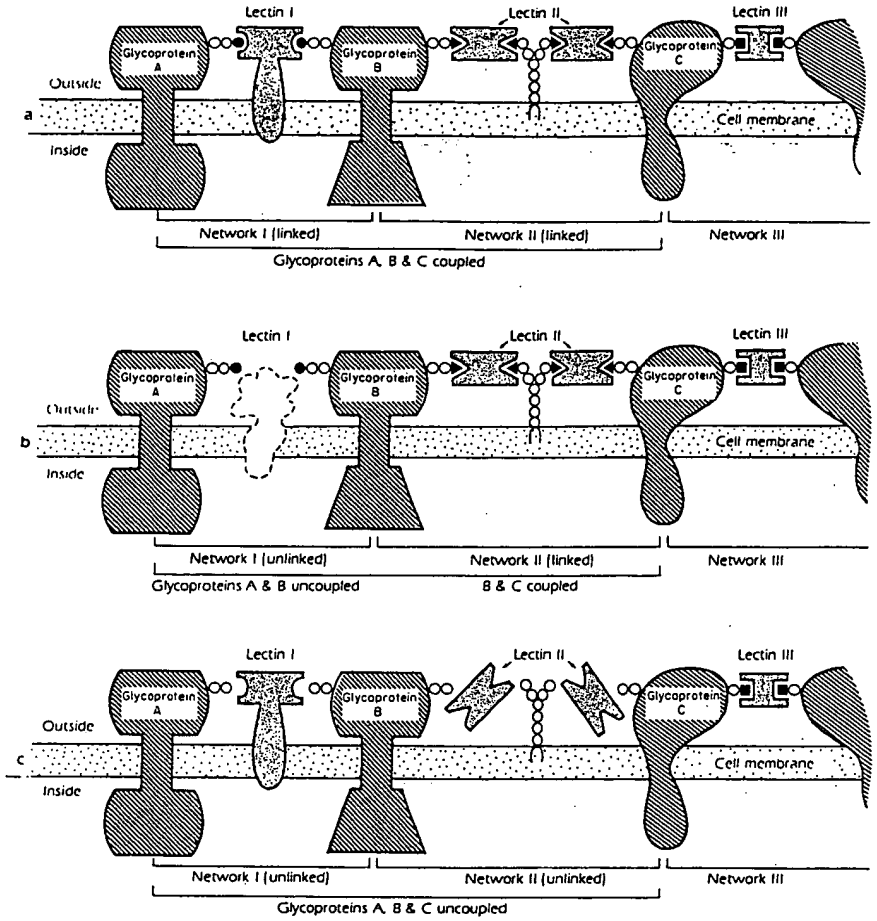

FIG. 1. Diagram of a cell membrane depicting a hypothetical network based on specific oligosaccharide–lectin interactions as a means of coupling different glycoprotein receptors. (a) Glycoproteins and glycolipids with like carbohydrate structures are linked by appropriate lectins. Linkage of glycoconjugates with different carbohydrate structures is achieved through the mediation of lectins of different binding specificities, e.g. glycoprotein A with glycoprotein C via networks I and II. (b) Network I is disrupted by the absence or inactivity of lectin I. (c) Networks I and II are both unlinked because the glycoconjugates are inappropriately glycosylated. Lectin I is represented as a membrane-associated protein, and lectins II and III are shown as soluble proteins; the three lectins have different oligosaccharide specificities. The lectins in this scheme might include microbial carbohydrate-binding proteins. Lectin–sugar networks need not be confined to plasma membranes but might operate in intracellular membranes and even in the cytosol. ○, ▲, ■, ●, monosaccharides linked in various ways; ∩, ceramide moiety of a glycolipid. (From Feizi & Childs 1987a).

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EGF and in their biological responses to added EGF. These observations suggest that a blood group A recognition system may be operating in this cell line. This requires further investigation.

A second series of relevant observations are those concerning the glycoprotein receptor for the insulin-like growth factor II (IGF-II). The first of these is the discovery from the molecular biology studies of Morgan et al (1987) that the IGF-II receptor and the cation-independent receptor for oligosaccharides bearing 6-phosphorylated mannose residues (Lobel et al 1987) are one and the same molecule. This topic is further discussed by Dr M. Czech (this volume). To accommodate these developments, an updated scheme for our proposed receptor 'cross-talk' mechanism is shown in Fig. 2 with a growth factor receptor (glycoprotein B) depicted as a multifunctional glycoprotein with a combining site for the growth factor and another for the sugar chains of those glycoproteins (e.g. glycoprotein A) with which it needs to communicate. We have proposed (Feizi & Childs 1987b) that such a mechanism, dependent on correct glycosylation, would be a versatile and adjustable means of communication between growth factor receptors and associated proteins in the same cells, and could explain many of the cooperative effects that have been observed among growth factor receptors.

These ideas should be testable in the near future, but first we need to have a sensitive means of identifying proteins that recognize the sugar chains of glycoproteins. Such a method has now been introduced and has given new information in several oligosaccharide recognition systems.

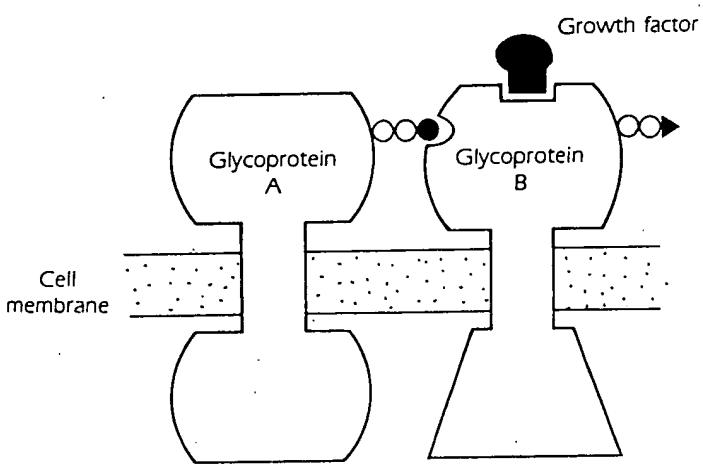
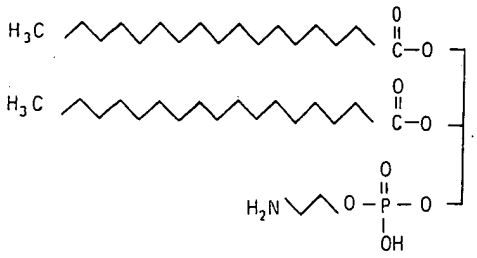


FIG. 2. Diagram depicting part of a hypothetical growth-regulating network involving oligosaccharide recognition. The growth factor receptor is shown here as a bifunctional glycoprotein (B) with a combining site for the growth factor and a 'lectin-like' site for an oligosaccharide structure that occurs on a second growth-regulatory glycoprotein (A). (From Feizi 1988).

Neoglycolipids as oligosaccharide probes for ligand-binding assays

The principle is to release oligosaccharides from glycoproteins, to conjugate these to a lipid and to use the resulting neoglycolipids as probes of oligosaccharide recognition after immobilization on insoluble matrices, e.g. silica gel or plastic plates, or after incorporation into liposomes (Tang et al 1985). Any oligosaccharide structure might be used in this system, including those synthesized chemically. Reduced oligosaccharides (such as those released from mucin-type glycoproteins by alkaline borohydride degradation) which are unreactive in conjugation reactions, can be converted into reactive oligosaccharide aldehydes by mild periodate oxidation (Tang et al 1985). For the idea and guidance in the oxidation experiments, I am indebted to Dr Y. C. Lee.

The lipid of choice was phosphatidylethanolamine dipalmitoyl (PPEADP, 1), because preliminary experiments have shown superior reactivities of conjugates using this lipid compared with those using several other readily available lipids. Conjugation is achieved by reductive amination, and the feasibility of this approach has been shown by the potent antigenicities of O-linked



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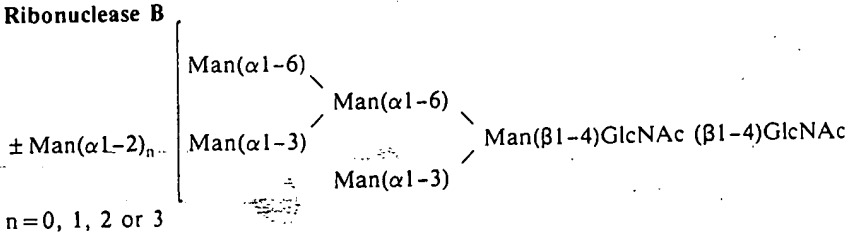
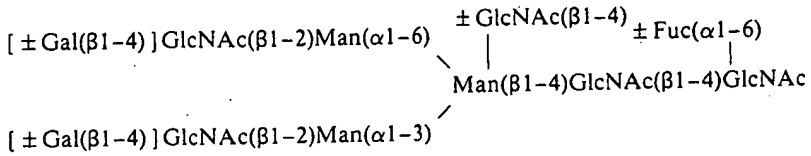
oligosaccharides released from mucin-type glycoproteins (Tang et al 1985) and from galactosyltransferase (Tang & Feizi 1987), and of sulphated oligosaccharides released from keratan sulphate by using endo-β-galactosidase (Tang et al 1986).

The conditions for conjugation of reducing oligosaccharides to PPEADP have been improved (Stoll et al 1988) and a series of neoglycolipids have been prepared from N-linked chains derived from structurally characterized glycoproteins (Mizuochi et al 1989), such as high mannose-type (oligomannosidic) oligosaccharides with five to eight mannose residues from ribonuclease B, and a spectrum of complex-type chains from human and mouse immunoglobulin G and from human transferrin. The neoglycolipids were chromatographed on silica gel plates. In collaboration with Dr A. M. Lawson, we have identified (Mizuochi et al 1989) individual neoglycolipid bands by liquid secondary ion mass spectrometry directly from the chromatograms using the procedure of Kushi & Handa (1985). The reactivities of the neoglycolipids with various carbohydrate-binding proteins are being investigated by overlay assays as used with natural glycosphingolipids (after Magnani et al 1980), as described below.

Ribonuclease B



Human IgG, mouse IgG and transferrin



Neoglycolipid overlays with the plant lectin concanavalin A and animal lectins

Growth modulation in animal cells by exogenously added plant lectins was mentioned earlier. As a first step in the study of lectins with growth-modulating properties, we assessed the reactivity of concanavalin A (Con A) with neoglycolipids derived from N-linked chains of glycoproteins (Stoll et al 1988). The specificity and sensitivity of this approach is illustrated for neoglycolipids from ribonuclease B (Fig. 3, panel I). The neoglycolipid mixture (2.5 μg total carbohydrate per lane) was chromatographed on silica gel plates and overlaid with ^{125}I-labelled Con A. Binding was detected by autoradiography (lane CA). The same lane was later stained by orcinol reagent to reveal the oligosaccharide bands (lane O). In accordance with previous knowledge, Con A bound to all four high mannose-type probes. This binding was specifically inhibited by α-methylmannoside (lane CA'). Also in accordance with previous knowledge, binding to biantennary oligosaccharide probes was weaker than to the high mannose-type probes. These results are described in detail in Childs et al (1989).

In collaboration with Dr K. Drickamer, we (Childs et al 1989) have investigated oligosaccharide recognition by the recombinant carbohydrate recognition domain of rat mannose-binding protein, rMBP-A (see Drickamer, this volume). This protein has been found to react preferentially with neoglycolipids from N-linked chains of the complex-type that have two non-reducing terminal *N*-acetylglucosamine residues. It reacts only weakly in the presence of a single non-reducing terminal galactose residue and with the high mannose-type oligosaccharides, and not at all with analogues that have two

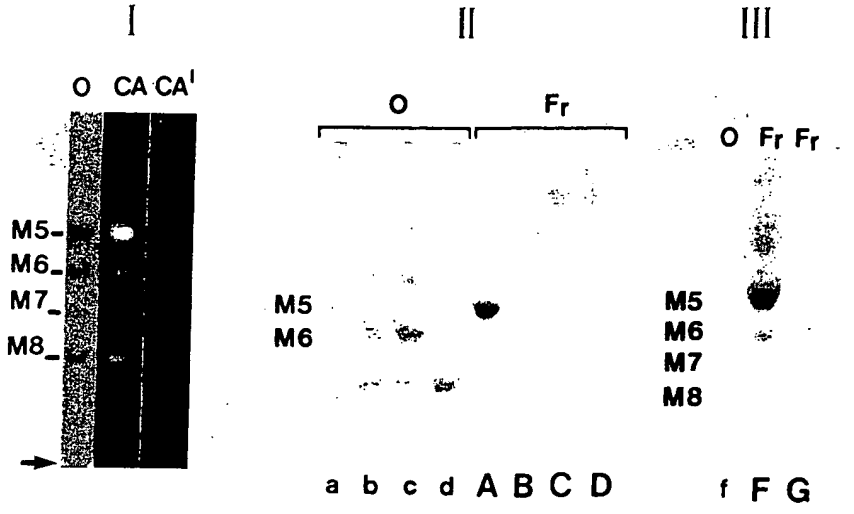


FIG. 3. Reactivities of the plant lectin Con A and of *E. coli* with neoglycolipids derived from glycoprotein oligosaccharides. Panel I, a mixture of neoglycolipids (2.5 μg carbohydrate per lane) derived from the oligosaccharides of ribonuclease B were chromatographed on silica gel plates using chloroform/methanol/water 105:100:28 (by volume) and overlaid with ^{125}I-labelled Con A. Lane CA shows binding of Con A detected by autoradiography and lane O shows the same lane after chemical staining with orcinol to reveal the neoglycolipid bands. Lane CA′ shows lack of Con A binding in the presence of 200 mM α-methylmannoside. Panel II, neoglycolipids (5 μg carbohydrate per lane) derived from the oligosaccharides of ribonuclease B (lanes A, a) and from sialidase-treated oligosaccharides of human and mouse IgG (lanes B, b and C, c respectively) and transferrin (lanes D, d) were chromatographed as above and overlaid with ^{14}C-labelled *E. coli* strain C600 isolated from a patient with urinary tract infection. Binding was detected by fluorography (Fr) and the same plate was stained with orcinol (O) to reveal the neoglycolipid bands. Panel III, ^{14}C-labelled *E. coli* were overlaid onto a chromatogram containing ribonuclease B oligosaccharides (10 μg carbohydrate per lane), in the absence (lane F) and in the presence (lane G) of 300 mM mannose. Lanes F and G show results of fluorography (Fr). Lane f shows the same lane as F stained with orcinol. Arrow indicates position of sample application. M_5 to M_8 indicate positions of the five high mannose-type oligosaccharide probes from ribonuclease B. Panel I from Stoll et al (1988) and panels II and III from Rosenstein et al (1988).

terminal galactose residues. Additional experiments with simple oligosaccharide probes have revealed a reactivity with non-reducing terminal fucose residues, as in the sequence Fuc(α1–4)[Gal(β1–3)]GlcNAc. Similar studies have been performed with natural mannose-binding proteins of rat and human sera, and with bovine serum conglutinin (collaborative studies with Dr T. Kawasaki, Professor P. J. Lachmann and Dr S. Thiel). The results (Childs et al 1989, Loveless et al 1989, Mizuochi et al 1989) indicate that the binding specificities of the

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TABLE 1 De of their neogly infection

Designation[a]	
Lactose	
LNT	
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LNDFH-I (Le^b)	
(A) tetra	
(A) hexa	C

[a]Blood group A, H
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four mammalian proteins are related, but not identical. Another common feature is that the recognition units for these proteins are small, possibly not much longer than monosaccharides.

Neoglycolipid overlays with *Escherichia coli*

The new technique using neoglycolipids lends itself well to studies of oligosaccharide recognition by microbial adhesins, particularly to identification of individual oligosaccharide structures among the diverse chains on glycoproteins. In studies with type 1 fimbriated *E. coli* from patients with urinary tract infections, specific adhesion has been visualized to selected neoglycolipids derived from glycoprotein oligosaccharides (Rosenstein et al 1988). Thus, when a series of oligosaccharide probes from ribonuclease B, human and mouse immunoglobulin G and from transferrin (5 μg carbohydrate per lane) were evaluated for reactivity with ^{14}C-labelled *E. coli* (Fig. 3, panel II), binding was detected to only the high mannose-type $Man_5GlcNAc_2$ (Man_5) probe (panel Fr, lane A). This is in accordance with previous studies (Firon et al 1982, Neeser et al 1986) which showed that cell attachment by type 1 fimbriated *E. coli* can be inhibited by free mannose and by certain oligosaccharides and glycopeptides having the non-reducing terminal sequence Man(α1–3)Man(β1–4)GlcNAc. This sequence is present on the Man_5

TABLE 1 Designations and structures of oligosaccharides, and the binding reactions of their neoglycolipids with *E. coli* strain C600 isolated from a patient with urinary tract infection

Designation[a]	*Oligosaccharide*	*Reactivity of neoglycolipid*
Lactose	Gal(β1–4)Glc	+ + + +
LNT	Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc	+ + + +
LNFP-I (H)	Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc / Fuc(α1–2)	+ +
LNDFH-I (Le^b)	Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc / | Fuc(α1–2) Fuc(α1–4)	+
(A) tetra	GalNAc(α1–3)Gal(β1–4)Glc | Fuc(α1–2)	+
(A) hexa	GalNAc(α1–3)Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc / Fuc(α1–2)	±

[a]Blood group A, H and Le^b activities, where present, are shown in parentheses. Taken from Rosenstein et al (1988).

structure and on some of the higher oligomannosyl structures (Man_6–Man_8) from ribonuclease B. The predicted faint reactivities with Man_6–Man_8 structures cannot be discerned in Fig. 3, panel II. However, when 10 μg carbohydrate material was applied per lane, as in Fig. 3, panel III, some binding to these higher structures was detected, and binding to all four bands was specifically inhibited in the presence of 300 mM mannose.

When a series of neoglycolipids derived from human milk oligosaccharides were used as probes in overlay experiments with several *E. coli* strains (Rosenstein et al 1988), a new adhesive specificity was revealed (Table 1). This adhesion is unrelated to the presence of type 1 fimbriae and appears to involve the lactose [Gal(β1–4)Glc]-related sequence in association with lipid, because it is not inhibited by free oligosaccharides. A related adhesive specificity has been observed among gonococci by Stromberg et al (1988) using a series of natural,

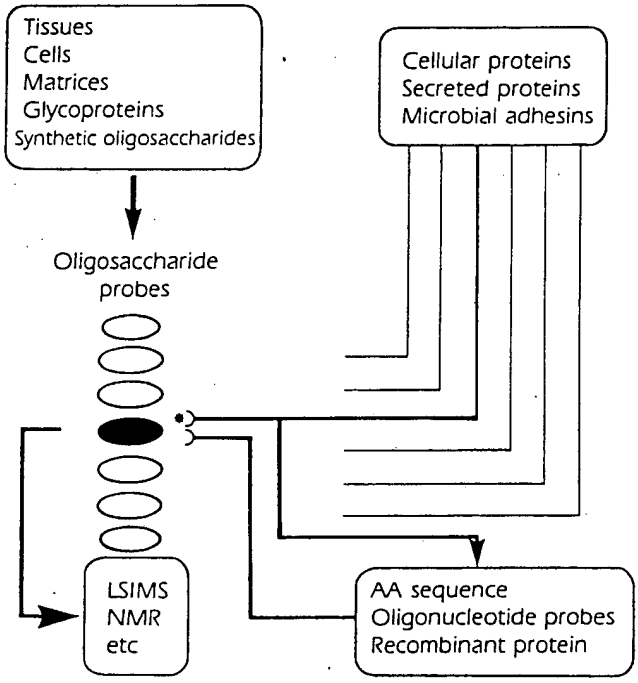

FIG. 4. A general strategy for elucidating oligosaccharide recognition systems. Whole tissues, isolated cells, extracellular matrices or individual glycoproteins, in addition to desired synthetic oligosaccharides, are used to generate neoglycolipids which are used in overlay experiments with radiolabelled proteins. Individual cellular or secreted proteins or microbial adhesins (asterisked) with carbohydrate recognition properties are singled out and characterized at the level of protein and gene structure. The oligosaccharides recognized are characterized by state-of-the-art methods of structural analysis (LSIMS, liquid secondary ion mass spectrometry; NMR, nuclear magnetic resonance spectroscopy).

epithelium-derived glycolipids. Since the lactose sequence is contained in the lipid-linked, membrane-associated domains of the majority of host cell glycolipids, we propose that this type of adhesive specificity may play an important rôle in the invasion of damaged epithelial cells, where the saccharide–lipid junction may be exposed.

When the lactose-containing sequence is modified by additional monosaccharides (including the blood group monosaccharides) binding of the *E. coli* is markedly impaired (Table 1). Thus it is predicted that the secretor gene and the genes coding for the blood group enzymes, or other glycosyltransferases whose levels change in epithelial cells during differentiation, proliferation and maturation, markedly influence binding and hence susceptibility to invasion.

There are many potential applications of neoglycolipids in the field of infectious diseases. We envisage banks of diverse oligosaccharide probes derived from natural sources and from synthetic oligosaccharides. These may be used to identify and map the oligosaccharide receptors for pathogens and their individual adhesins. Such studies could form the basis of novel drug designs targeted at the prevention of the initial events of infection.

Future perspectives

I wish to highlight the wide biomedical potential of this new method. Figure 4 shows a general strategy for elucidating novel oligosaccharide recognition systems in diverse settings. It is envisaged that, as appropriate, whole tissues, isolated cells, extracellular matrices as well as individual glycoproteins will be used as sources of oligosaccharide probes, and that these will be supplemented by probes derived from synthetic oligosaccharides. By the neoglycolipid overlay approach, it should be possible to identify specific carbohydrate-binding proteins from among cellular and secreted proteins and microbial proteins. The proteins that bind to particular neoglycolipid bands would be eluted, sequenced and eventually produced by protein molecular engineering. In parallel, the oligosaccharide recognition units would be identified by state-of-the-art analyses and new profiling methods under development by Drs E. F. Hounsell and M. S. Stoll and associates in our laboratory.

There is a great potential for neoglycolipids in the 'quality control' and selection of recombinant glycoproteins produced by molecular engineering for administration to man. It is well appreciated that particular sugar chains of glycoproteins are important determinants of the compatibility, the clearance rates and *in vivo* bioactivities of some glycoproteins. Strategies are envisaged, as illustrated in Fig. 5, whereby oligosaccharide probes from recombinant glycoproteins generated from alternative cellular sources are subjected to the

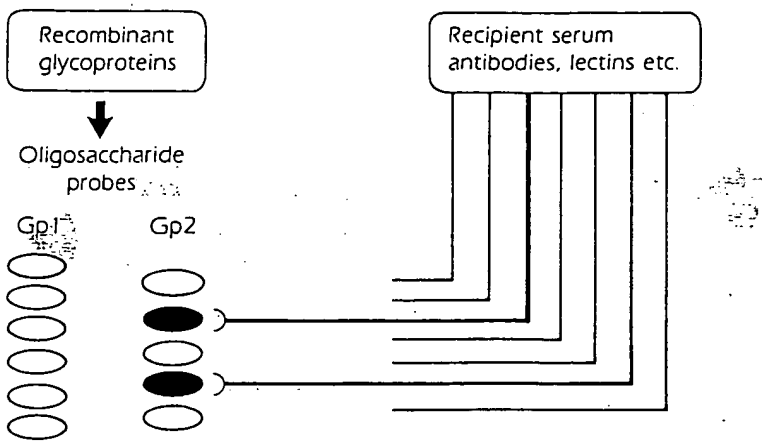

FIG. 5. A strategy for 'quality control' of the oligosaccharides of recombinant glycoproteins. Alternative glycoproteins, designated as Gp1 and Gp2, are assessed with respect to the compatibility of their oligosaccharides with antibodies in recipient sera and their reactivities with sequence-specific monoclonal antibodies and lectins, by neoglycolipid overlays. Thus incompatible oligosaccharides reactive with antibodies in recipient sera, or, conversely, oligosaccharides with desired structures, are specifically and conveniently identified.

new profiling methods which would be extended to include probing with recipient sera and with sequence-specific monoclonal antibodies and lectins. In this way, glycoproteins decorated with undesirable oligosaccharides could be readily identified and discontinued, and those with the desired structures accepted for large-scale production.

Acknowledgements

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DISCUSSION

Ruoslahti: Have you demonstrated that you can take a mixture of bacterial extract, do the overlay assay, and have it work specifically as illustrated in Fig. 4?

Feizi: So far we have worked with whole bacteria that have been metabolically labelled. If one were to make an extract, one could hope to identify the adhesive protein itself.

Hughes: The thin-layer chromatogram that had been overlaid with bacteria (Fig. 3, panel III) showed remarkable specificity for $Man_5GlcNAc_2$ (Man_5): binding to the $Man_6GlcNAc_2$ species was practically non-existent.

Feizi: This is according to prediction, because the non-substituted Man(α1–3)Man(β1–4)GlcNAc sequence would be present on only a subpopulation of the Man_6 structures (Liang et al 1980).

Hughes: Do you always find the same binding specificity in different strains of *E. coli*, or is there a range of bacterial strains or substrains with different $Man_nGlcNAc_2$ oligosaccharide-binding specificities?

Feizi: We have not studied that yet.

Sharon: I was delighted to see that you confirmed our findings and those of Neeser et al (1986) that Man_5 is a good inhibitor of cell attachment by type 1 fimbriated *E. coli*. In hapten inhibition experiments, Man_5 is about 20–30 times better than methyl α-mannoside (Firon et al 1983).

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We studied several strains of *E. coli*, mainly with hydrophobic glycosides which are strong inhibitors. We also looked at some strains with branched oligosaccharides, synthetic methyl glycosides prepared by T. Ogawa (Saitama, Japan); they did not have the $GlcNAc_2Asn$ structure. Neeser et al (1986) used different strains of *E. coli* and a different assay system. Although they found essentially the same results with Man_5, there were some differences with the Man_6 oligosaccharide. We also found that aromatic α-mannosides are powerful inhibitors of the mannose-specific *E. coli* lectin (Firon et al 1987). Our data seem to indicate that all *E. coli* that are 'mannose-specific' have the same specificity for different oligomannosides.

In most bacteria, the lectin or lectin-like proteins are on the outer surface, usually in the form of fimbriae. The fimbriae can be easily isolated by shaking the bacteria followed by differential centrifugation. The isolated fimbriae show the same pattern of carbohydrate specificity as the fimbriated bacteria. Interestingly, examination of the binding of purified fimbriae with different specificities, isolated from infectious strains of *E. coli*, to sections of human kidney (Korhonen et al 1986) and of rat brain (Parkkinen et al 1988) gave patterns supporting the widely held belief that binding of the parent bacteria to complementary cell surface sugars plays an important role during bacterial invasion.

Ruoslahti: Dr Feizi, have you tried to get mammalian cells to bind to, say, fibronectin, as we showed several years ago?

Feizi: We are preparing to do binding studies with whole cells.

Sharon: Dr Ruoslahti, you tried some years ago to overlay with mammalian cells proteins separated by polyacrylamide gel electrophoresis.

Ruoslahti: Yes, but that was with adhesion proteins, such as fibronectin and vitronectin. That works well. Thus, in principle one can do this with mammalian cells.

Sharon: One can do it with bacteria on polyacrylamide gels.

Fukuda: Dr Feizi, can you separate your neoglycolipid by HPLC as well?

Feizi: We are investigating that.

Fukuda: When you conjugate with reduced oligosaccharides you need to use periodate, which probably destroys the sialic acid.

Feizi: This is something we have been discussing with Dr Lee. With reduced oligosaccharides we need to refine the oxidation procedure to protect the sialic acid. I don't know whether we shall completely overcome this. We may have to be resigned to losing a proportion of the sialic acid residues.

Hart: Do your coupling procedures destroy O-acetylated sialic acid? At what pH is your reductive deamination done?

Feizi: It is virtually neutral. If the O-acetylated sialic acid were on a reducing oligosaccharide it would not be destroyed during conjugation. If it were on an O-linked chain, it would have been destroyed already in the course of releasing the chain from protein by the alkaline borohydride degradation procedure.

Watkins: Or released by hydrazinolysis, because this is done under alkaline conditions and leads to loss of both N- and O-acetyl groups.

Feizi: Yes; this also applies to N-linked chains released by hydrazinolysis. Such oligosaccharides should ideally be released using endoglycosidases.

Finne: Your result with the mannose-binding protein was perhaps unexpected. Is it possible that sometimes when you conjugate the oligosaccharides to the carrier lipid you change the conformation of the oligosaccharides in such a way that you get the 'wrong' result?

Feizi: That is something we have to consider. But so far we have not observed any spurious reactions using L-α-phosphatidylethanolamine dipalmitoyl (PPEADP).

Rademacher: The implication is that the mannose-binding protein is binding *N*-acetylglucosamine. Could you comment on the purification of those sugars? Human IgG contains a substantial amount of oligomannose which could co-run.

Feizi: It is most unlikely that the reactions we have observed with IgG oligosaccharides represent lectin binding to trace amounts of oligomannosidic (high mannose) oligosaccharides, because the oligomannosidic oligosaccharides from ribonuclease B reacted extremely weakly.

Rademacher: Was that binding confirmed by treatment with jack bean β-hexosaminidase?

Feizi: We have observed that while the lipid conjugates of oligosaccharides with terminal galactose residues derived from transferrin do not bind to the mannose-binding protein, the conjugates of the β-galactosidase-treated oligosaccharides bind strongly (Childs et al 1989).

Rademacher: Have you blotted a preparation of group A streptococci with the mannose-binding protein?

Feizi: No.

Rademacher: Have you blotted neoglycolipids using whole serum?

Feizi: Yes; for example, whole sera containing monoclonal antibodies have been used successfully (Tang et al 1985, 1986).

Rademacher: The assumption is that the monoclonal antibody is purely to the sugar and there is no peptide contibution to the binding. What is the minimum binding affinity detected by your method?

Feizi: We need to do proper measurements. At this stage, we can say that it is analogous to the minimum binding affinity required for immunofluorescence or Western blotting. The overall affinity is high because the ligands are presented in a multivalent state. With monovalent oligosaccharide ligands the affinity may be too low for binding in this type of assay. The virtue of this method is that the chromatography gives rise to clustering of the oligosaccharides. Reactivities with antibodies and lectins are strongly influenced by cooperative effects of multivalency.

Barondes: Can you use glycopeptides or are they too messy?

Feizi: We have not yet made neoglycolipids using glycopeptides.

Barondes: What is the largest oligosaccharide you can resolve?

Feizi: Up to a decasaccharide, so far.

Hughes: You have used the carbohydrate-binding domains of the lectin, as isolated by Kurt Drickamer. Might the specificity of the whole protein be different?

Feizi: I didn't go into this, but we have also worked with the whole mannose-binding protein from rat serum, and there is a subtle difference in reaction pattern (Childs et al 1989). It is not yet clear whether the collagen domain of the mature protein somehow influences the reactivity of the carbohydrate recognition domain, or whether there is more than one type of carbohydrate recognition domain in the natural protein; i.e. it is a heteropolymer, not a homopolymer.

Hughes: Kurt would have picked up the latter case in his sequence search.

Drickamer: It is known that there are two different genes that encode highly homologous mannose-binding proteins. It is not clear whether the natural protein is a mixture of homopolymers of the two different gene products, or a heteropolymer and, if it is the latter, what the proportions are in the circulation. Until one clarifies these issues, as Ten says, one can't distinguish any effect of the additional structure of the intact protein, not present in the recombinant binding domain, from the effect of the presence of several non-identical proteins.

Watkins: How do you think the endogenous lectins tune the response of the epidermal growth factor (EGF) receptor through an interaction with A determinants? Are you suggesting that there are endogenous *N*-acetylgalactosamine-binding lectins in group A individuals? Would that not be rather dangerous?

Feizi: I am suggesting that endogenous lectins (possibly those with blood group A specificities in A431 cells) might, for example, influence the extent of aggregation of the EGF receptor on the cell membrane and thus tune the level of responsiveness of the receptor glycoprotein to EGF. There is already a good example of an *N*-acetylgalactosamine-specific lectin—one of the so-called soluble β-galactoside-binding proteins in rat lung (Leffler & Barondes 1986). It reacts more strongly with blood group A structures than with the unsubstituted β-galactosyl backbone. Whether this lectin normally associates with the blood group A structures in intact membranes must be studied. Lectins of this type have all the hallmarks of cytoplasmic proteins; they lack the signal sequence found on secreted and membrane-associated proteins, although some people claim to have also detected them on the cell surface. Cells do break down and these are very stable proteins. It is therefore conceivable that the released proteins find their way to the cell surface. There may, of course, be other families of membrane-associated lectins yet to be discovered.

Watkins: If it were to have any physiological effect, the lectin would have to be fairly widely distributed.

Feizi: Soluble lectins of this family occur in most cell types.

Fukuda: We usually look at binding in solution. Your assay involves an interaction between solid-phase carbohydrate and the protein in solution. That could account for differences in the specificity of the interaction.

Feizi: The same reservation applies to overlay assays with natural glycolipids. It is true that we don't know how these structures are orientated on the silica gel plate.

Fukuda: It's nice to complement such experiments with studies using liposomes, which may be similar to the membrane.

Feizi: Binding assays using liposomes are rather messy. We are interested in incorporating the neoglycolipids into cells. That might be easier to work with.

Muramatsu: Can your reagents adhere to plastic disks? If so, how many experiments could you perform with 1 mg of oligosaccharide?

Feizi: Thousands of microtitre wells can be coated with neoglycolipids derived from that amount.

Kobata: I have a comment on the mannose-binding lectins of bacteria. We have analysed several glycoproteins purified from the epithelial cells of large intestine and found that none of them contains oligomannosidic oligosaccharides. The sugars are mostly highly branched, with elongated outer chains. So I wonder what that bacterial lectin is doing in the intestine, because it has no ligand on the epithelium.

Feizi: We studied *E. coli* from patients with severe urinary tract infections. These bacteria adhere to epithelial cells of the urinary tract.

Sharon: Dr Kobata, are there no hybrid-type oligosaccharides either?

Kobata: We could not find them.

Fukuda: We have some preliminary data on colonic cell glycopeptides. Poly-*N*-acetyllactosamine is the major type of oligosaccharide. There are no oligomannosidic oligosaccharides, but many O-linked oligosaccharides are present.

Hughes: Perhaps the 'mannose-binding' protein binds to poly-*N*-acetyllactosamine structures.

Sharon: Or perhaps the mannose-binding protein is really an *N*-acetylglucosamine-binding protein.

Feizi: The presence of non-reducing terminal galactose residues on complex-type oligosaccharides inhibits the binding of the rat lectin. Terminal *N*-acetylglucosamine seems to be the ligand of choice.

Hughes: It could be a poly-*N*-acetyllactosamine chain with *N*-acetylglucosamine at the non-reducing terminus.

Feizi: Branched poly-*N*-acetyllactosamine structures with terminal *N*-acetylglucosamine residues are certainly candidate ligands. We find that neoglycolipids

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derived from chitin oligosaccharides, such as chitobiose [GlcNAc(β1–4)GlcNAc], react well.

Finne: We observe a similar absence of oligomannosidic glycans in small intestinal epithelial cells (Finne et al 1989). It appears that we do not want to have *E. coli* that bind mannose in our intestinal tract.

Sharon: You really don't need much. The Tamm-Horsfall glycoprotein has only one oligomannose chain (Dall'Olio et al 1988) and yet it binds very well to mannose-specific *E. coli*. It may have a role in infection, for example as a vehicle for clearing bacteria from the urine.

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